Biological Fundamentals of AnM Techniques in Peanut Crop Production

FEI-FEI QIN

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Chapter 1

General Introduction

1.1 Research background

1.1.1 Botanical properties of peanut plant

Peanut (*Arachis hypogaea* L.) is a species in legume family Fabaceae. It is an annual herbaceous plant. The peanut plant is unusual because the flower is borne above ground and pods containing one to five seeds are produced underground. This is the difference from other legume species and therefore it is also called groundnut. With optimum soil moisture the seed of peanut swells, hypocotyl elongates and pushes out a pair of cotyledons and a root sprout. The hypocotyl elongates downward as the cotyledons are lifted upward. The cotyledons provide nutrients for the growth. The seed coat begins to dissolve and the nutrients inside are used up as the root develops out peripheral roots and root hairs. As most of the legumes, the elongation of the hypocotyl of peanut stops when the cotyledons are sent out of the soil and meet light. A pod (seeds contained in seed coat) of peanut begins in the yellow petaled flower, which is borne in auxiliary clusters aboveground. After self-pollination, the flowers fade and the stalk at the base of the ovary, which is called peg, elongates and turns downward and penetrates into the soil and later buries the pod (fruit) several inches deep in the ground to complete the development.

1.1.2 The problems with early and late pegs

In some cases, the cotyledons meet light from soil cracking even under the soil surface and make the hypocotyl to stop elongation with the cotyledon node remained under the soil surface. Therefore, the early two branches from the cotyledon node develop flowers and produce pods early. The early pods would rot before harvest and be infected by *Aspergillus flavus*, which produces aflatoxin and contaminates other later pods (Shen 1958; Shen and An 1988; Craufurd et al. 2006). The early pods may reduce the formation of the later pods by nutrient competition or by some kind of signal message that shows satisfaction with the existing seeds as offspring. The theoretically ideal type of peanut plant would be one that sets its young pods on the same day and spends the rest of the growing season to fill them (Duncan et al. 1978) but the plant cannot flower at the same time so that it is not possible for all pegs to penetrate into soil in a short period. Consequently, some pods from the early flowering would mature earlier in the soil than others, and these pods would risk rotting or preharvest aflatoxin contamination in underground conditions (Chemberlin et al. 2004; Craufurd et al. 2006). Moreover, the early pods also compete against the new flowers for carbohydrates. The flowers and the consequent pegs in later growth period are usually far from the soil

surface, especially for the varieties with standing types. If the pegs could not penetrate into soil within a specific period, there would be no enough time for full development of the pods. Therefore, at the agronomical level, some practices are needed to lift up the cotyledon node out of the soil surface, delay the elongation and penetration of the early pegs, and welcome the late pegs for earlier penetration.

1.1.3 Practices to solve problems of early and late pegs

1.1.3.1 Clearing the seedling and exposing the hypocotyl

When peanut seedlings well establish, the soil around the base part of a seedling is clawed and moved away, so that the hypocotyl is exposed with cotyledons left above the ground. This practice is called "clearing seedlings" in peanut cultivation and proves significant in improving peanut production (Shen 1958). By clearing the peanut seedlings, the cotyledon node is relatively far from the soil surface. The first pair of lateral branches from the cotyledon node grows robustly, where the pegs from the early flowers are also far from the soil surface and the too early penetration of the pegs is controlled. The photosynthetic carbohydrates are used for more effective branches and more productive flowers instead used for the development of the too early pods. The practice of "clearing seedlings" has been widely adopted in peanut production in China, especially in the Shandong Province, the main peanut production area.

1.1.3.2 The AnM and related techniques in peanut production

Most cultivars of peanut crops grown in Japan and China belong to *Arachis hypogaea*, the Virginia type, with standing or half-standing types instead crawling types. In many cases, the cotyledons meet light and the hypocotyl stops elongation when the light penetrates into the soil by soil cracks and irradiates the cotyledons, especially in drought conditions. In these cases, cotyledons remain under soil surface and the first pair of branches develops from the cotyledon node and suffers the adverse conditions such as low light and high humidity under soil surface. The early branches develop their flowers, which pollinate by themselves, develop pods and set seeds earlier than usual. The early pods have several disadvantages: 1) using up the carbohydrates produced by the young plant that has not developed enough and so inhibiting the plant development by nutrition competition; 2) inducing a signal to inform the plant that it has already had offspring and making the plant to be lazy or lose its crisis sense in further seeds producing; and 3) the ripen early pods would rot in soil, infected by fungi that producing aflatoxin, which in turn contaminates other pods (Shen and An 1988; Shen 1990; Chemberlin et al. 2004; Craufurd et al. 2006). Therefore, the peanut producers have tried many practices to lift up the cotyledon node out of the soil surface. As described above, "clearing soil around the seedling" is one of the practices (Shen 1958). In order to more effectively solve this problem in peanut production, Shen (Shen 1976; Shen and An 1988) proposed the so-called AnM technique, including first inducing extra-elongation of hypocotyl, exposing the elongated hypocotyl to light and dry air, and then at the later stage earthing up soils to welcome the late pegs. The letters, A, n,

and M, refer to the cross-section shapes of the ridge at different stages. The letter "A" shows the cross-section of the ridge after seeds are sown; the letter "n" shows the ridge cross-section at the seedling stage when soil around the hypocotyls are removed away and the hypocotyls are exposed; and the letter "M" shows the ridge cross-section at the full blossom stage when soil on both sides of the ridge is earthed up to welcome the late pegs. The practice is shown in Fig. 1.1 (Shen and An 1988).

In order to practice the AnM technique easily by machine in the field, Shen et al. (1996) again proposed a modified AnM technique in combination with film mulching. A shallow flat ridge is mulched with plastic film with small soil mound covered above the seed holes to induce extra-elongation of the hypocotyl. This was corresponding to the "A" stage of the AnM technique. At the "n" stage, the soil was removed away when the cotyledon node was sent out of the film surface. Unlike in the case of the basic AnM without film mulch, the soil in the ridge under the film surface was not removed any more. Two months after seeding, the soil in the furrows was earthed up to welcome the later pegs. This is the "M" stage treatment and similar to the case of basic AnM. It is comparatively easier to use machine to operate the modified AnM technique. The practices of the modified AnM are shown in Fig. 1.2. In mechanized practice, the soil mounds over the film surface are actually connected together as a small ridge.

1.1.3.3 Agronomical advantages of the AnM techniques

The AnM method lies in one principle that the cotyledons of peanut do not or do not completely emerge from the soil in most cases because the hypocotyl elongation stops when the cotyledons meet light (Angelo 1973; Hoshikawa 1980). Even in case of emergence from the soil, the internode of the cotyledons remains inside the soil. Therefore, branches from the cotyledon internode are usually covered by soils, resulting in a poor growth. By clearing the soil around the base part of the peanut seedling, the first pair of lateral branches grows robustly and the differentiation of flower buds is improved, resulting in more effective branches and more productive flowers (Shen and An 1988). At the "n" stage, clearing soil around the seedlings can also remove weeds and improve root penetration into the soil. Exposing the hypocotyl and young cotyledons into sun light and air with lower humidity is also a treatment of hardening the seedlings or the so-called xerophytophysiological practices (Xu 2007). Moreover, the pegs in the soil can directly absorb nutrients and water (Shen 1985) and seed filling would be improved if the N, P, Ca and S are supplied by dressing fertilization in combination with the practices of earthing up at the "M" stage (Shen 1976; Shen and An 1988). However, this knowledge has neither been well understood and the dressing fertilization has nor been adopted widely by peanut production although a 20% yield increase by dressing fertilization has been reported (Shen 1985). The modified AnM technique has proved the same effective in addition to the high yield improvement by film mulching techniques (Shen at al. 1996; Du et al. 2006). Although some agronomic advantages in AnM technique have been examined, there still remains a lot of blank

even in the yield increasing effect by AnM method.

1.1.4 Practices possibly related with the AnM techniques

1.1.4.1. Film mulching

In peanut cultivation, the technique of plastic mulching has been popular in peanut production regions in China. Plastic mulching improves peanut yield by 30%, through increasing soil temperature, reducing soil surface evaporation, and improving soil physical properties (Shen 1990; Shen et al. 1996). Plastic mulching has proved effective in improvements in absorption of nitrogen, phosphorus, potassium, calcium, sulfur and other nutrients (Wu et al. 2007). With film mulching, conditions of low humidity, mild dry soil and high soil temperature are created and these conditions are especially important in the humid and wet climate in Japan. Mulching reduces the need for tillage and the use of weed-control chemicals. Water is conserved because mulches reduce the evaporation of soil moisture. Water absorption by a mulched soil is greater than that of an unmulched soil. Mulch also prevents the formation of soil crusts. In addition, soil loss from heavy rain and wind is decreased. Therefore, an additive or synergistic effect may be expected from the combination of the AnM technique with film mulching.

1.1.4.2 Seedling transplanting

Seedling transplanting is used to fill the emergence gaps between seedlings and have not been used in large scales. Usually, 10-20% of emergence gap between seedlings is often observed in the summer peanut crops. However, using seedlings to fill the emergence gaps cannot compensate the loss enough. In central China, peanut crops are relay-intercropped into winter wheat to increase land use efficiency (Cai et al. 1996; Liang 1996; Zhang et al. 1996; Knörzer 2010). When the winter wheat is harvested, the peanut seedlings have already established. However, it is difficult to sow peanut between the dense rows of wheat before harvest. Therefore, researchers tried to transplant peanut seedlings after wheat harvest. They have fortunately found that the transplanted peanut crops show many advantages over the directly seeded ones, including better formation and development of nodules, less leaf spot disease and somehow yield increase (Hou and Meng 1996 at http://wuxizazhi.cnki.net/ Search/PEAN602.005.html).

1.1.5 Concept and application of xerophytophysiology and signal transduction in plant production

Xerophytophysiology is the study of physiological and biochemical functions of plants in adaptation to drought conditions, with not only soil water deficit but also low humidity, salinity and strong irradiations including the UV and blue light as the components of the strong irradiations (Xu 2007). In application of the mechanisms in xerophytophysiology to plant production, the “drought” is not necessarily the real drought that would damage the plants. Instead it is just a treatment to impose a stimulus to the plant and to produce signals in order to induce some internal adjustment that would benefit the plants. Among the manipulations of xerophytophysiological

applications, the examples include mesocotyl exposure for sorghum plants (Xu et al. 2009d), clove exposure for garlic plants (Qin et al. 2008a), partial root-zone drying for tomato (Xu et l. 2009b) and potato (Xu et al. 2011a), restricted irrigation for house tomato (Xu et al. 2010), blue light irradiation in canopy for tomato crops (Xu et al. 2012) and seedling drying for transplanted wheat plants (Xu et al. 2011c). In all the treatments of stimulation, the whole root, in some cases the partial root, is in the well moist soils and therefore there is no real drought or it is just a modest or false drought.

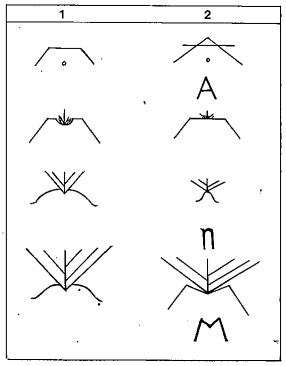

Fig. 1.1 Diagram of the AnM technique (1, the common ridge cultivation; 2, AnM. Reproduced according to Shen and An 1988).

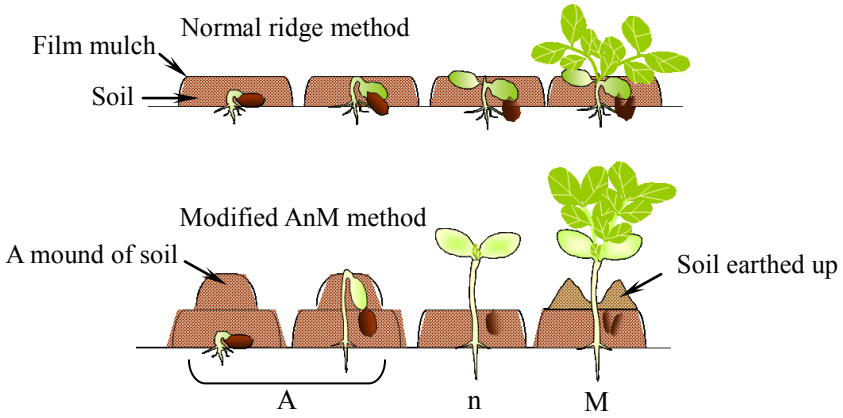

Fig. 1.2 Illustrate of the modified AnM (Upper: normal ridge method; Lower: Modified AnM).

1.2 Research objectives

1.2.1 Hypotheses

The practices of AnM cultivation include three steps: 1) the peanut seeds are sown a little deeper in the ridge to induce extra-elongation of the hypocotyls. At this time it is called “A” stage; 2) the elongated hypocotyl is exposed to light and lower humidity by removing the soil away around the seedling and at this time it is called “n” stage; and 3) soil in the furrows between ridges are earthed up to welcome the later pegs in order for pegs to penetrate into the soil properly. It is called “M” stage.

A hypothesis is proposed that the key step of the AnM method is the hypocotyl exposure at “n” stage. Exposing the hypocotyl to light and dry air is considered as one of applications of xerophytophysiology. When the hypocotyl, which usually remains under soil surface, is exposed to light and dry air, the peanut seedling perceives the light and drought stimulation even though there is no real drought stress, sends the signal to the internal gene system, where some stress-induced genes are activated, transcripted and expressed with the expected positive consequences of xerophytophysiological regulations.

1.2.2 Propositions

Now many research achievements in plant signal transduction in response to environmental stimulations have been obtained by the modern laboratory techniques at the level of molecular biology, especially using the model plant, *Arabidopsis* (Riechmann et al. 2000; Baluška et al. 2006; Jensen et al. 2008). However, this knowledge has not yet been changed to feasible techniques in scaled plant production. In the present research, the AnM technique in peanut production is considered as one of the applications of signal transduction in plant production. Some of the agronomic advantages in the AnM technique have been confirmed (Shen and An 1988; Shen 1990) and adopted in large scale in peanut production in China. However, the mechanisms for the crop improvements at levels of plant physiology and molecular biology have not been clarified. If the growth and pod yield are improved, the photosynthetic rate and/or the photosynthetic size (leaf area) must be increased. Therefore, improvements in photosynthesis and related processes should be confirmed to support the plant growth and yield improvements by the AnM technique. As suggested by Xu (2007), the main positive consequence of the xerophytophysiological responses is the increased leaf turgor potential caused by osmotic adjustment. Therefore, osmotic adjustment, which is usually induced by drought stimulation, and the related processes should be examined to see whether the AnM technique especially the hypocotyl exposure at “n” stage could induce such xerophytophysiological regulation.

Even in the production sites, the color pigmentation in the exposed hypocotyls can be observed. Usually, anthocyanin accumulation plays roles in the protection of photosynthetic apparatus and resistance to many abiotic and biotic stresses (Holton and Cornish 1995; Steyn et al. 2002) and anthocyanin itself acts as osmolyte and

accumulates together with other osmolytes such as sugars. Therefore, accumulation of anthocyanins and other osmolytes must be confirmed whether this contributes to the basis of physiological mechanisms by the hypocotyl exposure. As hypocotyl exposure is considered as drought stimulation, the ultimately, activation, transcription and expression of some drought related genes should be examined to confirm the mechanisms for the hypocotyl exposing stimulation-induced xerophytophysiological regulations at the molecular biological level.

1.2.3 Objectives

1.2.3.1 Confirmation of the agronomical advantages of the basic AnM technique

Although some agronomic advantages have been examined (Shen and An 1988), more experiments are needed to confirm whether the AnM technique is feasible in peanut production and the related physiological mechanisms should be examined for the yield increasing effect of the AnM technique in peanut production.

1.2.3.2 Confirmation of agronomical advantages of the modified AnM technique

It is wondered that practices of the basic AnM technique are complicated and unfeasible for mechanization. In one experiment of the present research, the modified AnM technique is confirmed in combination with mulching, which is suggested to be easily performed by machines including film mulching and soil placing equipment. The film mulching itself has proved effective in yield increasing in peanut production (Khan 2002). In another experiment, the modified AnM technique is re-designed in combination with a biodegradable black film in comparison with a transparent white film. The agronomic advantages of the modified AnM technique are confirmed in comparison with the basic AnM techniques and the related physiological mechanisms were clarified.

1.2.3.3 Seedling transplanting as the alternative of AnM technique

Both the basic and modified AnM techniques are labor-cost or unfeasible for mechanization. Therefore, in one experiment of the present research, transplanting of seedlings with elongated hypocotyls is developed as an alternative of AnM techniques and comparisons are made between seedlings with and without extra-elongated hypocotyls and between seedling transplanting and direct seeding. The agronomical performances including pod yield and analysis of plant growth dynamics and physiological fundamentals including photosynthetic activities and osmotic adjustment are examined.

1.2.3.4 Confirmation of improvements in photosynthetic activities by AnM techniques in basic, modified and seedling transplanting alternative manners

Since the plant growth and yield are closely related with leaf photosynthesis, therefore, improvements in photosynthesis and related processes should be confirmed to support the plant growth and yield improvements by the AnM technique. The photosynthesis-light response curve, the photosynthetic hysteresis and the stomatal oscillation are also analyzed to confirm the improvements in photosynthetic activities.

1.2.3.5 Analysis of the osmotic adjustment as one of the consequences of xerophytophysiological regulations

As suggested by Xu (2007), osmotic adjustment is the main consequence of the xerophytophysiological regulations. Osmotic adjustment results in increased leaf turgor potential, which is the driving force for plant growth and photosynthetic stomatal opening. Osmotic adjustment can be easily analyzed by the pressure-volume (P-V) curve method. In the present research, the P-V curve analysis is used in all experiments including the basic, modified and transplanting-alternative AnM techniques.

1.2.3.6 Analysis of the leaf water retention ability by the excised leaf transpiration declining curve

It is complicated to determine the leaf surface morphological structure directly. The leaf water retention ability is considered proportional to leaf surface morphological structure. Therefore, in the present research, the analysis of the leaf water retention ability by the excised leaf transpiration declining curve is adopted in all experiments.

1.2.3.7 Analyses of the antioxidant enzymes

Plant cells are protected against oxidative stress by antioxidant enzymes. The main antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). In cells of plants in adverse environmental conditions such as drought, extreme temperatures and excess and strong irradiation, reactive oxygen species (ROS) increases with the degree of stresses. The antioxidant enzymes are responsive to clean up the increased ROS (Alscher et al. 2002; Gill and Tuteja 2010). Measuring these enzyme activities may provides an easy and precise way to understand an important part of the defense system, which is expected to contribute a healthy crop and product quality improvements in addition to defense against stresses. In the present research, the hypocotyl exposure, although it is not a real stress, might induce ROS increase and antioxidant enzyme activations. Therefore, it is necessary to examine the antioxidant enzymes to confirm the xerophytophysiological responses caused by the hypocotyl exposure.

1.2.3.8 Measurements of the concentrations of anthocyanins and other osmolytes

In response to hypocotyl exposing stimulation, osmolytes such as sugars and anthocyanins might actively increase to cope with the process of osmotic adjustment. Anthocyanins do not only act as one of the osmolytes but also protect the cells from injure by the stresses (Holton and Cornish 1995; Steyn et al. 2002). Anthocyanin accumulation is also one of the indications of the xerophytophysiological regulations. Therefore, in the present research, concentrations of anthocyanins and other osmolytes in leaves and hypocotyls of the peanut seedlings are examined with their hypocotyls exposed. The relations of accumulations of anthocyanins and sugars with the osmotic adjustment are discussed.

1.2.3.9 Analysis of the drought responsive gene

One of the drought response gene, *Gdi-15* (groundnut desiccation induced), is shown

also to be homologous to the enzyme (flavonol 3-O-glucosyltranferase) which involved in anthocyanin biosynthesis (Gopalakrishna et al. 2001). In the present research, activation, transcription and expression of *Gdi-15* gene are ultimately examined to confirm the mechanisms for the hypocotyl exposing stimulation-induced xerophytophysiological regulations at the molecular biological level.

Chapter 2

Physiological and Yield Responses to the Basic AnM Technique in Peanut Production

2.1 Abstract

In sustainable crop production, the most important is a healthy crop resistant to pests and adverse environments. One of the approaches to a healthier crop is through hardening the crop seedlings based on the mechanisms of xerophytophysiology. One of the hardening practices, the so-called AnM cultivation method for peanut production, was tested in the present research. The three letters, A, n, and M, refer to the shapes of the ridge at different stages. The letter "A" shows the shape of ridge cross-section after the seeds were sown; the small letter "n" shows the ridge cross-section shape at the seedling stage, when the hypocotyls are exposed to light by removing away the soil around; and the letter "M" shows the ridge cross-section shape at the full blossom stage, when soil is earthed up from both sides of the ridge to welcome the pegs. Peanut (*Arachis hypogaea* L.) was grown in experimental field of a fine-textured Andosol. After the hypocotyls were exposed for two weeks, the plants appeared more vigorous with a shortened and stronger type. After earthing up treatment, both plant growth rate and leaf photosynthetic activity increased, consequently resulting in a shell yield increase of 20%. According to the P-V curve analysis, the fraction of the symplastic water was larger, the osmotic concentration was higher and as a consequence the leaf turgor potential became higher in leaves of hypocotyl exposed peanut plants, especially at the middle and later stages when the soil was earthed up to the ridge. The high leaf turgor maintenance is considered as the main one of the mechanisms for the yield increasing effect of the AnM treatment.

Key words: AnM method, hardening, osmotic adjustment, peanut (*Arachis hypogaea* L.), turgor potential, water fraction.

2.2 Introduction

Research in plant environmental stresses used to emphasize harmful effects on growth of the plants and many efforts have been made how to fight the stresses such as water deficit on the basis of breeding resistant varieties and finding suitable production systems (Aspelmeier and Leuschner 2004; Condon et al. 2004). At the cost of millions of scientists' work and tremendous amounts of research fund, hundreds of genes that are induced under drought have been identified and, a range of measures, from gene expression patterns to the use of transgenic plants, are being used to study the specific function of these genes and their role in plant drought resistance (Cushman and Bohnert 2000; Chaves et al. 2003; Bray 2004; Villalobos et al. 2004). Actually, some very beneficial effects of such stresses as mild water deficit and lower temperatures are expected by slowly increasing stresses and inducing internal physiological adjustments and regulations that protect plants from the damage when the later severe environmental stresses are suddenly imposed (Munns 1988; Blum 1997; Ruiz-Sánchez et al. 2000; Sharp et al. 2004; Izanloo et al. 2008; Navarro et al. 2008;). Plant seedlings previously exposed to water stress often undergo less inhibition of growth when water stress is severe in later stages, and grow better in case there is no water stress than do plants not previously exposed to such stress. This is called "hardening" based on the mechanisms of xerophytophysiology (Xu 2007). The terminology "xerophytophysiology" is a new compound word, with "xero" meaning drought adapted and so "xerophyte" the drought adapted plant (Xu 2007). Here, "xerophytophysiological adjustment and hardening" means hardening plants by placing plant seedlings under modest or partial drought conditions, and many processes inside plant change in response to the imposed drought signals. One of the changes is osmotic adjustment whereby the solutes concentration in the tissue or cells becomes higher than usual (Chen and Jiang 2010). Morphologically, the plant grows thicker and shorter with more wax and cuticle deposited on leaf surface (Curtis et al. 1996). Both physiologically and morphologically, the plant becomes healthier than usual and consequently shows better growth and higher resistance to diseases and stresses in later growth periods. At the level of molecular biology, it is associated with signal transduction (Ramanjulu and Bartels 2002; Bañon et al. 2004; Inoue et al. 2008; Shao et al. 2008a). The seedling perceives the adverse environmental changes and transits the signal to the internal and changes including the related gene expressions and metabolisms occur in response to the signaling with the consequence of increased ability to adapt the adverse environment. Physiologically, the response is associated with osmotic adjustment that involves accumulation of osmotically active substances and the consequent maintenance of leaf turgor potential, which is the driving force for growth and stomatal opening. Morphologically, better root growth and higher root: shoot ratio, stronger epicuticular protective layer and many other healthier traits are induced. In crop production, the succeeded xerophytophysiological practices include 1)

the restricted or deficit irrigations such as the so-called partial root zone drying (PRD) (Wakrim et al. 2005; Liu et al. 2006; Shao et al. 2008p), infiltration irrigation (Li et al. 2005; Warrick and Lazarovitch 2007), and sub-irrigation (Patel et al. 1999; Crossley 2004; Xu et al. 2011b) for fruits such as grapes and oranges and fruit vegetables such as tomato; 2) mesocotyl exposition for sorghum (Xu et al. 2009d); 3) low humidity for greenhouse crops (Xu et al. 2007); and 4) UV or blue light irradiation to peach fruit during the maturity (Rapparini et al. 1999).

Peanut or groundnut (*Arachis hypogaea* L.) is a valuable cash crop and produced in large scale in China, India and the United States. Most of the crop is cultivated where the average rainfall is in a range of 600 to 1200 mm and mean daily temperature is higher than 20˚C. In the peanut production areas in China, farmers used to hardening the peanut seedlings by removing the soil away around the base part of the seedlings with part of the hypocotyl and roots exposed. The seedlings get shorter, thicker and healthier than usual. Diseases are also avoided by good aeration and low humidity around the base part of the seedling. This measure has been adopted by almost of the farmers because it results in improvement of yield and resistance to diseases and adverse environments. In Chinese language this measure is called “Qingke” and means “clearing the seedlings by removing away the surface soil around the hypocotyl” (Shen 1958). The so-called AnM method is a modification of “Qingke” based on the special reproductive biology of peanut. The seed-containing pods of peanut mature underground instead of aerially as in most of other legumes. The flowering cannot occur at the same time or during a short period. Consequently, some pods from the early flowering would mature earlier in the soil than others and these pods would risk rotting or preharvest aflatoxin contamination in underground condition (Shen 1990; Shen and An 1988; Cole 1989). Moreover, the early pods also compete against the new flower for carbohydrates. Therefore, some researchers have taken measures to prevent the early pegs (the pollinated female flowers) from early penetration into the soil, or delay the penetration of the early pollinated pods into the soil. The AnM method is also one of the measures for early peg control in addition to benefit from the mechanisms of “Qingke” (Shen 1976; Shen and An 1988). The three letters, A, n, and M, refer to the shapes of the ridge at different stages. The letter “A” shows shape of the cross-section of the ridge after the seeds are sown; the small letter “n” shows the ridge shape at the seedling stage, when the hypocotyls are exposed by removing away the soil around; and the letter “M” shows the ridge shape at the full blossom stage, when soil is earthed up from both sides of the ridge to welcome the pegs.

Agronomic advantages by Shen and his group (Shen 1985; Shen and An 1988) showed a yield increase by the AnM method. However, this knowledge and the related physiological basis for the yield increase have not been well understood since almost thirty years ago. Here, a hypothesis was proposed that AnM method would be a practice of applications of xerophytophysiology in plant production. And clearing seedlings by

removing away the soil around with hypocotyls exposed to light at “n” stage might be the key for the hardening effect caused by osmotic adjustment. Therefore, the AnM method was confirmed, with not only the agronomic traits and photosynthetic activities, but also the parameters related to osmotic adjustment and turgor maintenance examined in the present study.

Moreover, the pegs in the soil can directly absorb nutrients and water (Shen 1958). Seed filling would be improved if the N, P, Ca and S are sufficient in the soil around the pegs (Shen 1985). This dressing fertilization has not been adopted widely by peanut production although a 20% yield increase by dressing fertilization has been reported (Shen 1990). Therefore, in the present experiment, an organic fertilizer was applied before the earthing-up treatment to confirm the effect of dressing fertilization.

2.3 Materials and Methods

2.3.1 Plant materials and treatments

Peanut (*Arachis hypogaea* L. cv. "Lainong-14") was grown on the organic experimental farm close to Matsumoto city in Nagano Prefecture, Japan. The soil was a fine-textured Andosol, with chemical properties as follows: total carbon-51 g kg^{-1}, total N-3.8 g kg^{-1}, NH_4^+-N-11.9 g kg^{-1}, NO_3^--N-15.6 g kg^{-1}, P-74 mg kg^{-1}, K-0.86 g kg^{-1}, Ca-1.87 g kg^{-1}, Mg-0.24 g kg^{-1}, Na-2.7 mg kg^{-1}, and CEC (cations exchange capacity)-218 meq kg^{-1} with a C/N ratio of 13. Seeds were sown in a ridge in middle May. As shown in Fig. 2.1, at this time of seed sown, the shape of the ridge cross-section was like the letter "A". The distance between the seed and peak of the ridge was 8 cm. Two weeks after seeds sown, the soil around the seedlings was removed away with the hypocotyl exposed to light. At this time, the shape of the cross-section of the ridge was like the letter "n". At the stage of full blossom, the soil at the bottom of the ridge was earthed up to welcome the pegs. At this time, the shape of the cross-section of the ridge was like the letter "M". The ridge was 20 cm high and the space was 30 cm between two plants and 60 cm between two ridges.

The experiment was designed as 4×4 Latin square with four treatments: 1) control, only ridging; 2) ridging with organic top-dressing fertilization; 3) AnM; and 4) AnM with organic top-dressing fertilization.

A n M

Fig. 2.1 The peanut AnM cultivation.

2.3.2 Fertilization

In this experiment, an organic fertilizer (N-52, P-30 and K-20 g kg^{-1}) fermented using oil mill sludge, rice bran and fish meal as materials was applied 200 g m^{-2} as base fertilization two weeks before sowing and 50 g m^{-2} as dressing fertilization two weeks before the "M" stage of earthing up treatment. As dressing fertilization, the same amount of fertilizer was applied to the soil surface in the control plot without hypocotyl exposing and soil earthing up treatment.

2.3.3 Photosynthesis measurement

A fully expanded youngest leaf of the third branch from the uppermost was used for photosynthetic measurement in the field four weeks after the earthing up treatment at

"M" stage. The measurement was done using LI-6400 portable photosynthesis system (LI-COR, Co., Ltd., Lincoln, Nebraska, USA). The leaf was measured at different photosynthetic photon flux (*PPF*) from 0 through 150, 250, 400, 600, 800, 1200 and 1600 to 2000 μmol m^{-2} s^{-1}. The maxium waiting time for *PPF* change was 3 min. The conditions in the leaf chamber were maintained with a relative humidity at 60±5% fluctuated according to transpiration and with air temperature at 22±1.5˚C fluctuated a little according to the light intensity. The net photosynthetic rate (P_N) at each set *PPF* was automatically read. The photosynthesis-light response curve was plotted against light intensity (*PPF*) and net photosynthetic rate (P_N) was modeled as $P_N = P_C(1-e^{-KI})-R_D$ according to Xu et al. (1994). P_C is photosynthetic capacity; R_D is respiration rate; K is the half time constant with the maximum quantum yield (Y_Q) defined as $Y_Q = KP_C$; and I is the *PPF*.

2.3.4 Analysis of osmotic adjustment

Osmotic adjustment was analyzed according to the Pressure-Volume (P-V) Curve method (Tyree and Hammel 1972; Turner 1988) with modification. Before measurement, the top part of the main stem was excised under water and rehydrated with the cut trace in water to saturation at temperature of 15˚C in the dark to prevent possible biomass loss from respiration. Here, it is important to cut the leaf under water and otherwise air can enter the xylem and disrupt the continuum of water flow in the leaf, which, if any, is called xylem cavitation or embolism. After the leaf was rehydrated to fully turgid status, the leaf was cleaned to remove the water attached to the leaf surface, and then set in a pressure chamber (Model 3000, Soil Moisture Ltd., Santa Barbara, California, USA). The exposed cut end of the leaf blade was about 1 cm beyond the rubber stopper and covered with a pre-weighed Eppendorf vial filled with tissue paper to absorb the expressed sap. Sap expressions were made successively by increasing pressure at 0.3 MPa per increment. This was done until the water potential reached a level of about 3.0 MPa. Air pressure in the chamber was increased until liquid just visibly wet the cut end of the xylem, which was viewed with a hand-held magnifying lens. The reading of the balanced pressure was taken as the absolute value of the negative water potential. The expressed sap in each increment was immediately weighed. After a series of measurements, the leaf was weighed, and then dried in an oven at 80˚C for 48 h.

The P-V curve was obtained by plotting the reciprocal of the pressure against the expressed water volume, on a relative basis, at each increment and was modeled as follow, $-\Psi^1 = \{\Psi_{FT}^{-1} - \pi_{s+a}^{-1}[\zeta_o - \beta(1-\zeta) - \zeta_{ap}]\}e^{-\alpha(1-\zeta)} + \pi_{s+a}^{-1}[\zeta_o - \beta(1-\zeta) - \zeta_{ap}]$.

Here, Ψ is leaf water potential at a level of leaf water content; Ψ_{FT} is leaf water potential at fully turgid status; π_{s+a} is osmotic potential in symplastic solution theoretically diluted by apoplastic water, i.e., average osmotic potential for whole leaf tissue water, at fully turgid status; ζ_{FT} is leaf water content at fully turgid status with a value of 1; ζ is leaf water content and $(1-\zeta)$ is leaf water deficit as the variable in this

equation; β is the constant showing apoplastic water changes; α is the constant showing the symplastic water change; and ζ_{ap} is apoplastic water fraction and shown as $\zeta_{ap} = \zeta_{FT}-\zeta_{sym} = (1-\zeta_{sym})$, where, ζ_{sym} is the symplastic water fraction.

The osmotic potential was modeled by $-\pi^{-1} = \pi_{s+a}^{-1} [\zeta_o-\beta(1-\zeta)-\zeta_{ap}]$. As shown in Fig. 2.2, the point where this linear curve of osmotic potential meets the exponential curve of water potential is theoretically the point of incipient plasmolysis or zero turgor point. In some cases, the linear curve and the exponential curve reach closer and closer but do not cross until the $(1-\zeta)$ reaches a large value. Therefore, the incipient plasmolysis point is defined as the point (y, x), i.e., $[(-\Psi^{-1})_{IP}, (1-\zeta)_{IP}]$, where $y = (-\pi^{-1}+\delta) = (-\Psi^{-1}-\delta)$ $(\delta=0.01y)$.

The point where the linear line crosses with the abscissa is the separatrix between symplastic (ζ_{ap}) and apoplastic (ζ_{sym}) water fractions.

The solute concentrations (C_S) at the fully turgid status and at incipient plasmolysis were calculated using the Van't Hoff relation (Jones 1992) as follows: $\pi = -RTC_S$ or $C_S = -\pi/RT$, where R is the gas constant and T is absolute temperature with a value for RT of 2437 J mol^{-1}. Therefore, the total cell sap solute concentration at 1 MPa is $-(-106/2437) \approx 410$ osmol m^{-3}.

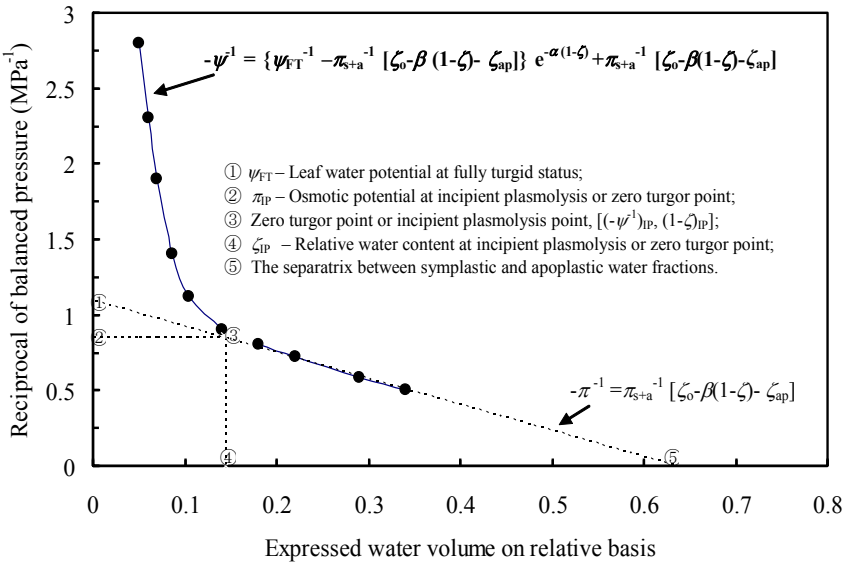

Fig. 2.2 A model of the Pressure-Volume curve.

2.4 Results

2.4.1 Peanut seedlings were hardened by hypocotyl exposure at "n" stage

Peanut seedlings were treated with hypocotyl exposed to light by removing the soil away after the extra-elongated hypocotyl was induced at the "A" stage. And this hypocotyl exposure treatment at the "n" stage lasted for almost one month before the earthing up treatment at the "M" stage which was supposed to be the key point for AnM method. Therefore, the plant growth was examined just before the earthing up to show the hardening effect by hypocotyl exposure (Table 2.1). The aboveground part of the peanut plants was slightly shortened with the hypocotyl exposing treatment at the early stages. However, the plants appeared more vigorous with leaf color in dark green (shown by the SPAD value). The base part of the shoot shown by diameter in AnM method after the hypocotyl exposing treatment at "n" stage became thicker than in the control. At the time just before soil earthing up treatment, plants already developed more pegs in AnM plots than in the control plots. The root system, as shown by root/total dry mass ratio, was more developed after hypocotyl exposure in AnM plots. In general, the plants were hardened by the hypocotyl exposure at "n" stage and looked stronger than those in the control.

Table 2.1 Plant examination of peanut just before soil earthing up treatment at "M" stage.

Treat.	Branch No.	Peg No.	Diam. (mm)	L. area (cm^2)	L. color (SPAD)	----------Dry mass (g)-------- Leaf	Stem	Root	Total	Root/Total
AnM	12.5^{ns}	16.2^{**}	7.8^{**}	111.6^{*}	49^{*}	5.2^{*}	4.5^{ns}	1.3^{*}	11.0^{*}	0.130^{*}
Control	12.3	12.5	6.7	103.3	43	4.8	4.3	1.0	10.1	0.100

Diam., the diameter of the base part the shoot; L. area, leaf area; L. color, leaf color; Root/Total, root/total dry mass ratio. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

2.4.2 Biomass production and the final shell yield were increased by the AnM technique

At the harvest time, irrespective of top-dressing fertilization before the "M" stage, the AnM technique increased plant biomass production and the final shell yield with a higher harvest index (Table 2.2). The AnM technique resulted in a higher ratio of marketable shells, suggesting a shell quality improvement by the AnM method. In AnM treatment, both the shell number and the average shell size (single shell weight) were higher compared with the control.

2.4.3 Dressing fertilization combined with the AnM technique increased plant biomass production and the final shell yield

Dry mass of shell, shoot and the total plant was all increased by dressing fertilization combined with AnM (Table 2.2). The harvest index decreased because the shoot growth was promoted more than the shell growth. Shell number per m^2 was but the shell size was not increased by the dressing fertilization. The percentage of marketable shell was decreased by the dressing fertilization because more small and/or hollow late pods formed on the promoted vegetative shoot.

Table 2.2 Dry matter production and fruit yield of peanut plants grown under different cultivation practices.

Treatment	Dry mass (g m^{-2})			Harvest index	Shell			
	Shell	Shoot	Total	(%)	No. (m^{-2})	Size (g $shell^{-1}$)	Yield (g m^{-2})	Marketable (%)
Control	269	510	781	34.6	135	2.24	303	84
Control-F.	281	594	876	32.1	144	2.33	335	79
AnM	322	480	802	40.1	143	2.62	374	87
AnM-F.	361	552	912	39.5	154	2.68	412	83
Fertilization	*	**	*	*	*	ns	*	*
AnM	**	*	ns	**	ns	**	*	**
F.×AnM	*	ns	ns	ns	*	ns	ns	ns

Control, only ridging; Control-F., ridging with organic top-dressing fertilization; AnM-F., AnM with organic top-dressing fertilization. Harvest index, the ratio of shell dry mass to plant total dry mass; Market (%), the percentage of marketable shells. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

2.4.4 Photosynthetic activities was increased by AnM treatment, but showed no positive effect by top-dressing fertilization

Leaf photosynthesis was promoted by the AnM treatment with increased photosynthetic capacity (P_C), dark respiration rate (R_D) and maximum quantum yield (Y_Q), both before and after earthing up treatment (Table 2.3). Dressing fertilization decreased P_C in plots without AnM treatment and showed no difference when AnM treatment was imposed (Table 2.3). Dressing fertilization also decreased the maximum quantum yield (Y_Q). As usual, in a given certain range, quantum use efficiency is positively proportional to the chlorophyll content or leaf green color. In the present experiment, AnM treatment increased the leaf green color but the side dressing fertilization did not (Data were not shown).

Table 2.3 Photosynthetic capacity (P_C), dark respiration rate (R_D), time constant (K) of light-response curve and the maximum quantum yield (Y_Q) before and one month after earthing up treatment at "M" stage of peanut leaves grown under different cultivation practices.

Treatment	----------(μmol m^{-2} s^{-1})----------				-------(mol mol^{-1})----	
	--------- P_C ----------		---------- R_D --------		---------- Y_Q----------	
	Before	After	Before	After	Before	After
Control	12.1	14.8	2.37	2.82	0.030	0.042
Control-F.		13.5		2.54		0.030
AnM	14.2	15.3	2.64	3.49	0.038	0.051
AnM-F.		15.6		2.79		0.037
Fertilization		*		ns		*
AnM	*	*	*	*	**	*
F.×AnM		*		*		*

Control, only ridging; Control-F., ridging with organic top-dressing fertilization; AnM-F., AnM with organic top-dressing fertilization. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

2.4.5 Osmotic adjustment was induced by AnM treatment

***Leaf turgor maintenance and improvement*:** In field conditions, leaf water potential (Ψ) was decreased slightly by exposing the hypocotyl during the early period of the "n" stage, but leaf turgor potential (P) was maintained slightly higher because the osmotic potential (π) was lower (Table 2.4). After the soil earthing up treatment, leaf water potential (Ψ) recovered but the osmotic potential (π) was still lower and therefore a higher leaf turgor potential (P) was maintained in peanut plants with AnM treatment.

Symplastic water fraction: Cell water is divided by the cell membrane into symplastic water (ζ_{sym}) and apoplastic water (ζ_{apo}) fractions. Symplastic water (ζ_{sym}) is favorable for biochemical metabolism and apoplastic water (ζ_{apo}) is not directly related with biochemical activities. Hypocotyl exposure increased ζ_{sym} in leaves of peanut plants and the effect lasted to the "M" stage after earthing up treatment (Table 2.4).

***Osmotic adjustment*:** Hypocotyl exposure at the "n" stage lowered the osmotic potential at the fully turgid status (π_{FT}) (Table 2.5). The leaf turgor potential at the fully turgid status (P_{FT}) became higher, especially at the "M" stage after the soil was earthed up to the ridge, although the water potential at the fully turgid status (Ψ_{FT}) showed no difference. The osmotic concentration at the fully turgid status (C_{FT}) was higher in the leaf of the hypocotyl exposing treated plants. This suggested that osmotic adjustment occurred and peanut plants withheld more osmotic solutes in their leaf cell sap in the adaptation to hardening process of the hypocotyl exposing treatment. The ability of osmotic ability can be shown by net increase of osmotically active solutes, the difference in C_{FT} between the treatment and the control. For example, the difference in C_{FT} between the AnM treatment and the control was 60 (548-488) osmol m^{-3}. This effect by the hypocotyl exposure could last to the "M" stage of earthing up treatment, e.g. more minus π_{FT}, and higher P_{FT} and C_{FT}. However, the top-dressing fertilization showed no significant difference.

***Osmotic potential and relative leaf water content at the incipient plasmolysis*:** The osmotic potential (π_{IP}) and the relative leaf water content (ζ_{IP}) at the incipient plasmolysis were both lower in response to both the hypocotyl exposure at the "n" stage and the earthing up at the "M" stage (Table 2.5). A lower π_{IP} or lower ζ_{IP} is usually important for environmental stress resistance.

Table 2.4 Leaf water potential (Ψ), Turgor potential (P) and symplastic (ζ_{sym}) and apoplastic (ζ_{apo}) water fractions in leaves of peanut grown under AnM treatment before and one month after earthing up treatment.

Treatment	Ψ (Mpa)		π (Mpa)		P (Mpa)		ζ_{sym}		ζ_{apo}	
	Before	After	Before	After	Before	After	Before	After	Before	After
Control	-0.57	-0.52	-1.13	-1.24	0.56	0.72	0.759	0.777	0.241	0.223
Control-F.		-0.50		-1.23		0.73		0.763		0.231
AnM	-0.64	-0.55	-1.22	-1.36	0.58	0.81	0.793	0.818	0.207	0.182
AnM-F.		-0.53		-1.33		0.80		0.803		0.197
Fertilization		ns		ns		ns		ns		ns
AnM	*	*	*	**	*	**	*	*	*	*
F.×AnM		ns		ns		ns		ns		ns

Control, only ridging; Control-F., ridging with organic top-dressing fertilization; AnM-F., AnM with organic top-dressing fertilization. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

Table 2.5 Leaf osmotic potential at fully turgid status (π_{FT}), leaf osmotic solute concentration at fully turgid status (C_{FT}), leaf osmotic potential at incipient plasmolysis status (π_{IP}), and the leaf relative water content at incipient plasmolysis status (ζ_{IP}).

Treatment	Ψ_{FT} (MPa)		π_{FT} (MPa)		P_{FT} (MPa)		C_{FT} (osmol m^{-3})		π_{IP} (MPa)		ζ_{IP}	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Control	-0.19	-0.20	-1.10	-1.19	0.91	0.99	451	488	-1.61	-1.56	0.799	0.803
Control-F.		-0.19		-1.22		1.03		500		-1.59		0.809
AnM	-0.20	-0.21	-1.18	-1.33	0.98	1.12	484	545	-1.67	-1.73	0.787	0.778
AnM-F.		-0.21		-1.29		1.08		529		-1.69		0.793
Fertilization		ns		ns		ns		ns		ns		ns
AnM	ns	ns	*	**	*	**	*	**	*	*	*	*
F.×AnM		ns		ns		*		ns		ns		ns

Control, only ridging; Control-F., ridging with organic top-dressing fertilization; AnM-F., AnM with organic top-dressing fertilization. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

2.5 Discussion

In the present study, extra-elongation of the hypocotyls of the germinating peanut seeds was induced by deep sowing at the “A” stage. Seedlings were hardened by the hypocotyl exposure at the “n” stage. And then soil earthing up was made at the “M” stage to welcome the pegs, when the plants looked stronger and healthier than those in the control. The biomass production and the final shell yield were increased by the AnM treatment (Table 2.2).

In order to elucidate the mechanisms of yield increasing effect caused by the AnM treatment, osmotic adjustment was analyzed after the “n” stage of hypocotyl exposure and the “M” stage of earthing up treatment. Osmotic adjustment and the consequent improvement of leaf turgor potential were induced without causing real water stresses. Another consequence of osmotic adjustment was the increase in symplastic water fraction or decrease in apoplastic water fraction (Tables 2.4 and 2.5). Inward water flow from apoplast to symplast might be caused by the increase in osmotic concentration inside the symplast. As usual, especially in dryland conditions, larger symplastic water fraction or smaller apoplastic water fraction means higher physiological activities (Jones 1992; Xu et al. 2000). In the present experiment, the larger symplastic water fraction was resulted from the hardening by the hypocotyl exposure. The peanut plants withheld more osmotic solutes in their leaf cell sap in the adaptation to the hardening process of the hypocotyl exposing treatment. And this hardening effect caused by hypocotyl exposure could last for almost one month and became more effective combined with the earthing up treatment at the “M” stage. Therefore, the leaf turgor potential (P) became higher, especially at the “M” stage when the soil was earthed up to the ridge. The high leaf turgor potential resulted in high photosynthetic activities and, as a consequence, a high final shell yield. The osmotic potential at the incipient plasmolysis (π_{lP}) was lower in leaves of hypocotyl exposing treatment. The π_{lP} is usually important for environmental stress resistance. The high leaf turgor maintenance is considered as the main one of the mechanisms for the yield increasing effect of the AnM treatment.

The AnM cultivation has proved effective in China for many years. The yield increasing effect is resulted from not only the hardening effect but also the peg growth control at the early stages (Shen and An 1988). It is known that fruiting and seed filling processes of peanut take place in underground conditions. Moreover, the flowering and pollinating processes can also take place in underground conditions in case that the early flowers from the base part of the shoot are covered by soils. However, this underground process adversely affects the shell yield of peanut. The early set fruit will be filled and ripe early, develop with only one seed, and in many cases rot in soil before harvest (Shibuya 1936). Not only do the early pods compete for nutrition and carbohydrates with the aboveground growth and fruit setting processes but also the rotted fruit results in postharvest aflatoxin contamination of the fruit. It is ideal if the

fruit set would take place within a short period or in one day and the seeds would be filled evenly during the later growth periods (Duncan et al. 1978). Usually, it is not possible for all pegs to penetrate into the soil in one day. However, growth and penetration of the early pegs can be controlled by removing away the soil around the hypocotyl and, as in some cases, by plastic mulching (Shen et al. 1996). Even when the flowering cannot take place in one day, the penetration of the pegs into the soil can be controlled within a short period. The growth of pegs is greatly controlled by air humidity, with a growth rate of 0.62 cm per day at a saturated air humidity but only 0.02 cm per day when the relative humidity lowers down to 57% (Shibuya 1936; Lee et al. 1972; Shen and An 1988). When the soil is removed away from the hypocotyl, dry air flows into the space and inhibits the elongation of the early pegs, resulting in penetration of most pegs together into the soil within a short period. This is the key point of the effect of treatment of soil removal shown by the small letter "n". Practically, the treatment of removing the soil away around the hypocotyl has long been a practice in peanut cultivation in China, even when the ridge is not adopted. This soil removal treatment can result in a remarkable yield increase (Shen 1990). The later pegs from the upper part of the shoot are comparatively far from the soil surface. The treatment of earthing up the soil makes the soil closer to the pegs and enables the pegs penetrate into soil following the early pegs from the lower part of the shoot. This is the key point of the effect of earthing up with the type shown by the letter "M".

The key point of the ridge type shown by the capital letter "A" lies in the principle that the cotyledons of peanut do not or do not completely emerge from the soil in most cases because the hypocotyl elongation stops when the cotyledons meet light (Angelo 1973; Hoshikawa 1980). Even in case of emergence from the soil, the internode of the cotyledons remains inside the soil (Shen 1990). Therefore, branches from the cotyledon internode are usually covered by soil, resulting in a poor growth. Moreover, the early flower buds would be covered by soils caused from aside plowing and pollinated inside the soil, resulting in poor pod setting and single seed pods. These underground pollinated pods ripe early and rot inside the soil. Therefore, peanut yield is limited by the characteristics of cotyledon internode remaining inside soil. In order to solve this problem, seeds of peanut are sown a little deeper than normal, about 8 cm, under the soil surface as shown by the letter "A" in the present experiment. The elongation of the hypocotyl does not stop until the cotyledons meet light. So, the deep sowing enables the hypocotyl elongate enough, and then the topsoil is removed away from around the cotyledon internode before emergence. When the seedlings emerge completely, the soil removal away from around the hypocotyl is done and the cotyledon internode is completely aboveground. This is the key point of the treatment shown by the ridge type "A". In other words, the point of "A" type treatment is for inducing the hypocotyl elongation. In case of plastic sheet mulched cultivation, seeds are sown under the sheet and covered with a small soil mound above the sheet (Shen et al. 1996). The elongation

is induced by the soil covering above the mulch. The yield can increase 27% by this treatment only.

The pegs and the fruit of peanut in the soil can directly absorb nutrients and water (Shibuya 1936) and seed filling would be improved if the nutrients are sufficient in the soil around the pegs. In the present experiment, in control only ridging without soil earthing up, top-dressing fertilization did not significantly increase pod yield although it increased the shoot dry mass (Table 2.2). However, the top-dressing fertilization with the organic fertilizer significantly increased shell yield and total dry mass production in the AnM treatment plots. In both control and AnM treatments, top-dressing fertilization increased shoot dry mass more than shell dry mass, resulting in a lower harvest index. The applied organic fertilizer was enrolled into the soil by the earthing up treatment and placed around the area where the pods grew. This might make the fertilization more effective than without soil earthing up treatment, where even the fertilizer was applied in the same amount.

Japan is a humid country with an annual rainfall more than 1200 mm in most areas. Peanut crops are usually grown in plain field bed without ridges. In many cases, soil wet conditions adversely affect the plant growth and pod yield of peanut. With the AnM cultivation method, problems caused by wet soil conditions can be solved because the ridges avoid the water accumulated in the soil and in the field. The hardening induced by hypocotyl exposure avoids the vegetative overgrowth. Therefore, in addition to the merits of the AnM treatments mentioned in the above paragraphs, the AnM method could show advantages in the specific humid conditions in Japan. In dryland conditions in countries such as China, the hardening, as shown by the osmotic adjustment and increases in turgor potential, could help the drought resistance in later dry months. In conclusion, the AnM cultivation method is effective in peanut production under various conditions.

Chapter 3

Physiological Fundamentals for the Modified AnM Cultivation Technique in Peanut Production

3.1 Abstract

The so-called AnM technique has been adopted in peanut production with the hypocotyls exposed to light to induce the xerophytophysiological regulations through signal transduction. A modified AnM technique in combination with film mulching was tested in the present experiment and the related physiological mechanisms were clarified in terms of osmotic adjustment. The ridge was covered with a kind of biodegradable film and peanut seeds were sown into the holes 3 cm deep under the film. The surface of plastic film on the seeded ridge was covered with 7 cm high of soil to induce more elongation of the hypocotyls. The soil on the film was removed after the cotyledonary node was sent out of the film surface. This is called modified or simplified AnM technique in peanut cultivation. A 2×2 factorial experiment was designed with film mulching (M) as the main factor and the AnM cultivation (A) as the sub-factor as follows: (1) MA, the ridge was mulched by film with AnM practiced; (2) MC, the ridge was mulched without AnM treatment; (3) NA, AnM was practiced without film mulching; and (4) NC, neither AnM or film mulching was practiced. Leaf photosynthetic measurement, plant growth analysis, pressure-volume curve analysis and excised leaf water retention analysis were done. Both AnM treatment and film mulching induced osmotic adjustment, as shown by the higher osmotic concentration and higher water fraction in the cell symplasm, and the consequent improvements in leaf turgor, leaf photosynthetic activities, plant growth and the final seed yield as well as disease resistance. In conclusion, the modified AnM technique was more effective than the basic AnM.

Keywords: Modified AnM, osmotic adjustment, peanut (*Arachis hypogaea* L.), signal transduction, xerophytophysiology.

3.2 Introduction

The AnM cultivation technique was first proposed by Shen (Shen and An 1988). The practices of AnM cultivation include three steps. First, at the "A" stage, the peanut seeds are sown a little deeper than usual in the ridge to induce extra-elongation of the hypocotyl. The second, at the "n" stage, the hypocotyl elongated more than usual is exposed to light and low air with lower humidity by removing the soil away around the young seedling just after the emergence. The third, at the "M" stage, soil in the furrows between ridges was earthed up to welcome the later pegs in order for pegs to penetrate into the soil properly. This method was adopted in large scale as a series of technique in peanut production in China. In the previous experiment in the Chapter 2, the yield increasing effect has been confirmed in terms of agronomic and physiologic advantages.

For most of the legumes including peanut, the elongation of the hypocotyl stops when the cotyledons are sent out of the soil and meet light. In some cases of peanut, when the cotyledons meet light, the hypocotyl stops elongation and leaves the cotyledonary node under the soil surface. Therefore, the early two branches from the cotyledonary node produce pods earlier and the early pods would be infected by *Aspergillus flavus*, which produces aflatoxin and contaminates other later pods. The early pods may reduce the formation of the later pods by nutrient competition or by some kind of signal message that shows satisfaction with the existing seeds as offspring. In AnM method, the treatments of deep sowing at the "A" stage and the extra-elongated hypocotyl exposure at the "n" stage solved the problem for early fruiting. As shown by the results in Chapter 2, a hypothesis is proposed, the main positive effect of the hypocotyl exposure is the xerophytophysiological responses caused by the stimulations of light and dry air. Usually, the hypocotyl remains under moist soil without meeting light and the dry air. When the hypocotyl is exposed to light and dry air, the plant perceives the environmental changes, produces the signal chemicals, and sends the signal to the internal gene system where some genes are activated and the related physiological and biochemical processes are regulated or adjusted. One of the main consequences is osmotic adjustment, which improves plant growth and photosynthetic activities by maintaining a higher leaf turgor potential, especially when the stimulation passes away.

The elongation of the early pegs is inhibited by decreasing the IAA and GA_3 concentration and increasing the concentration of ABA of gynophore (Zhang et al. 2004). Moreover, inhibition of elongation of early pegs promotes flower bud differentiation for the later pegs and helps form strong seedling. If the elongation of the early pegs was controlled, the carbohydrates and nutrients would be used for development of the later pegs. The additional dressing fertilization of compound fertilizers, organic fertilizers or special nutrients such as calcium and silicon could contribute to the yield increasing as shown in previous experiment in Chapter 2, by

performing at the same time with the practice of the soil earthing up at the “M” stage.

It is wondered that practices of the basic AnM technique are complicated and unfeasible for mechanization. Later, Shen et al. (1996) proposed the new technique of AnM cultivation method in combination with plastic-film mulching. The surface of plastic films on the seeded ridge is covered with a mound of soil to induce more elongation of the hypocotyls instead of the deep sowing in the basic AnM. The soil on the film is removed after the cotyledonary node is sent out of the film surface. This is called modified or simplified AnM peanut cultivation. The modified AnM technique can be easily performed by machines including film mulching and soil placing equipment. It has proved that film mulching plays an important role in yield improvement, weed control and pest controls (Wang and Han 1986; Zhen et al. 2005). Plastic film mulch significantly increases soil temperature, plough layer moisture and available soil nutrients (De et al. 2005; Zhen et al. 2005; Ramakrishna et al. 2006). Therefore, the AnM practice in combination with film mulching is considered as a promising technology in peanut production. However, the physiological fundamentals of the modified AnM cultivation in peanut production have not been clarified. Therefore, in the present study, the modified AnM technique was re-designed in combination with mulching using biodegradable black film. The effects of the modified AnM peanut cultivation technique in improvements of plant growth, yield and disease resistance were confirmed in climatic conditions in Japan. In addition, the physiological fundamentals of the modified AnM technique in improvements of plant growth and yield were clarified in terms of xerophytophysiological responses.

3.3 Materials and Methods

3.3.1 Experimental site

The experiment was conducted in 2009 in the farm fields located in Matsumoto highland plain area (36°12′N, 137°48′E; 700 m above sea). This area is characterized by the central highlands climate with less precipitation than other coast areas in Japan. The details of the climate data are shown in Table 3.1.

Table 3.1 Climate data in the Matsumoto highland plain area, Nagano Prefecture, Japan (http://www.jma-net.go.jp/nagano).

Climate variables	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year
Average high (°C)	4.9	5.6	10.0	17.5	22.6	25.5	29.0	30.5	25.0	19.0	13.3	7.9	17.6
Average low (°C)	-5.5	-5.3	-2.0	3.9	9.4	14.8	18.9	19.8	15.3	7.9	1.7	-3.1	6.3
Precipitation (mm)	31.1	42.5	73.5	86.8	92.5	135.9	132.6	95.8	162.3	89.4	52.9	23.3	1018.8

3.3.2 Soil properties and fertilization

The experiment was conducted in polyethylene rainout shelters. The experimental field under the rainout shelters has been used and managed in organic ways without chemical fertilizers and pesticides for more than 20 years. The soil in the experimental field belongs to Andosol, a volcano ash, with the properties as shown in Table 3.2. Two weeks before sowing, a bio-fertilizer fermented using rice bran, oil mill sludge and fish meal as materials was applied 200 g m^{-2} on dry mass base. The bio-fertilizer contained N, P and K at 40.2, 28.3 and 19.2 g kg^{-1}, respectively.

Table 3.2 The chemical properties of the Andosol soil.

pH	EC	total C	total N	NH_4-N	NO_3-N	P	K	Ca	Mg	CEC
(H_2O)	(mS cm^{-1})	------(g kg^{-1})-------		----------(mg kg^{-1})----------						(cmol kg^{-1})
5.9	0.04	37.1	2.9	6.1	4.9	166.0	326.0	2845.0	429.0	18.1

EC, electrical conductivity; CEC, cation exchange capacity.

3.3.3 Experiment design

A 2×2 factorial design was adopted with film mulching treatment as the main factor and the AnM cultivation as the sub-factor as follows: (1) MA, the ridge was mulched by a bio-degradable black film with AnM practiced; (2) MC, the ridge was mulched but AnM was not practiced; (3) NA, AnM was practiced without film mulching; and (4) NC, Neither AnM nor film mulching was practiced. The plots were arranged in a 4×4 Latin Square design with each shelter containing 4 plots. The ridge was 10 cm high and the space was 20 cm between two plants and 80 cm between two ridges.

3.3.4 The basic AnM treatment

The basic AnM referred to Chapter 2. In the present experiment, the peanut seeds

(*Arachis hypogaea* L. cv. Chibahandachi) were sown on May 15 and the soil around the hypocotyl was removed on May 25. Soil on both sides of the ridge was earthed up on July 15 and harvest was done on September 10.

3.3.5 The modified AnM treatment

The modified AnM technique is performed in combination with film mulching. A shallow flat ridge was made and mulched with a bio-degradable black film. On May 15, the peanut seeds were sown 3 cm deep into the holes and then the holes were covered with soil of 7 cm high. The covered soil was used to induce more elongation of the hypocotyl, which then in turn sent the cotyledonary node out of the film surface. This was corresponding to the "A" stage of basic AnM. At the "n" stage, the soil was removed away when the cotyledonary node was sent out of the film surface. Unlike in the case of the basic AnM without film mulch, the soil in the ridge under the film surface was not removed any more. Two months after seeding, the soil in the furrows was earthed up to welcome the later pegs. This was the "M" stage treatment and similar to the case of basic AnM.

3.3.6 Plant management and analysis of plant growth dynamics

The peanut plants were irrigated properly according to the evapotranspiration demand by a spray system over the crop and under the roof of the rainout shelter. Plants were sampled every 5-10 days, dried in oven at 85˚C, and used for the growth curve analysis. The dry mass per plant was plotted against the growing time (day) and a mathematic equation was used to model the growth dynamic curve according to Qin et al. (2008b) as follows: $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$, where, G_M was the maximum biomass increment; G_B was the original biomass of the plant at the beginning; α was the constant related with the steep part of the curve; β was the constant related with sloping part of the curve before and after the fast growth; τ was the time point when the biomass increment reached half amount of G_M; and t was the growth time (day).

3.3.7 Measurement of leaf photosynthesis and leaf color

Measurement and analysis of leaf photosynthesis are described in details in Chapter 2. The photosynthetic light response curve was analyzed by an exponential equation as $P_N = P_C(1-e^{-KI}) - R_D$, where P_C was photosynthetic capacity; R_D was respiration rate; K was the half time constant with the maximum quantum yield (Y_Q) defined as $Y_Q = KP_C$, and I is the *PPF*. The *PPF* at compensation point (PPF_C) was defined as *PPF* at P_N=0.

After the photosynthetic measurement, the leaf color was measured using a chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Konica Minolta Sensing, Inc., Tokyo, Japan). The readings by the non-destructive, quick and easy measurement indicate the chlorophyll content of the plant leaves.

3.3.8 Evaluation of disease index

The percentage infection of the plants was calculated from 20 plants in each treatment. Disease index (DI) for the brown leaf spot caused by *Mycospaerella arachidis* was estimated as DI = ∑(Number of infected leaves to a certain degree ×

Degree coefficient)/(total sample leaf number × highest degree coefficient). The degree coefficient was scored from 0 (no symptom) through 1 (12.5% of the leaf area was infected), 2 (25%), 3 (50%) and 4 (75%) to 5 (completely infected). The rotting shells, which might be contaminated by aflatoxin, were also recorded.

3.3.9 Evaluation of osmotic adjustment ability by the pressure-volume curve analysis

Osmotic adjustment was analyzed according to the Pressure-Volume (P-V) Curve method (Tyree and Hammel 1972; Turner 1988) and the derails are described in Chapter 2. The P-V curve was modified as follow:

$-\Psi^{1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}])$,

where, Ψ was leaf water potential at a level of relative leaf water content (ζ); Ψ_{FT} was Ψ at full turgor or in saturated leaf water conditions; π_{s+a} was the osmotic potential (π) in the symplastic solution theoretically diluted by apoplastic water; (1-ζ) was leaf water deficit as the independent variable in this equation; β was a coefficient used to adjust the slope of the less sloping part of the curve; α was a coefficient associated with the slope of steeply sloping part of the curve.

3.3.10 Measurement of midday leaf water potential and determinations of midday osmotic potential and midday turgor potential

After the measurement of photosynthesis, the water potential of the second fully-expanded leaf from top of the main shoot was measured during 12:00-13:00 using the pressure chamber technique (Model 3000, Soil Moisture Ltd., Santa Barbara, California, USA) according to the method by Turner (1988). This was defined as midday leaf water potential (Ψ_{MD}) to show the leaf water status at the most stressful time during a day. The corresponding midday leaf osmotic potential (π_{MD}) was estimated from the equation of pressure-volume curve using the data of midday leaf water potential (Ψ_{MD}). Midday leaf turgor potential (P_{MD}) was calculated as the difference between Ψ_{MD} and π_{MD}. It is meaningful if a leaf can maintain high turgor potential for plant growth and stomatal opening at midday time when the light energy is saturated and the evapotranspiration may impose stresses to the plant. That is why leaf water potential and turgor potential are used for evaluation of the plant physiological activities.

3.3.11 Analysis of the excised leaf transpiration curve

The measurement of leaf water retention ability was done according to the existing method (Clarke and McCaig 1982; Quisenberry et al. 1982; Xu et al. 1995) with modifications. The fully expanded youngest leaf from the uppermost was excised under water at nightfall and the cut end was placed in water with the leaf blade in air at saturated vapor pressure in a bucket in dark at 15˚C, to have the leaf rehydrated over night to a fully-turgid state. The leaf sample was then placed under light in air saturated with vapor in a closed acrylic container for 30 min to allow the stomata to open and then the leaf was placed on a net fixed on a light paper box under 500 μmol m^{-2} s^{-1} of

PPF at 60 ± 5% of relative humidity and 22±2˚C of temperature. The leaf sample was weighed at 2-15 min intervals using an electronic balance. Changes in fresh weight were recorded and water loss was determined on a relative basis. A curve was obtained by plotting leaf relative water content against time, called the excised leaf transpiration curve (Fig. 3.1) (Xu et al. 2009c) as follow: $\zeta=[\zeta_{sat}-\zeta_{SC}(1-\beta t)]e^{-\alpha t}+\zeta_{SC}(1-\beta t)$, where, ζ was relative water content of the excised leaf; ζ_{sat} was the leaf relative water content at saturation before drying started; ζ_{SC} was the leaf relative water content at the time when the stomata closed; t was the time from beginning of the transpiration course; β is the constant related with the slope of the second gently-sloping phase of the curve; and α was the constant showing the slope of the first steeply-sloping phase of the curve.

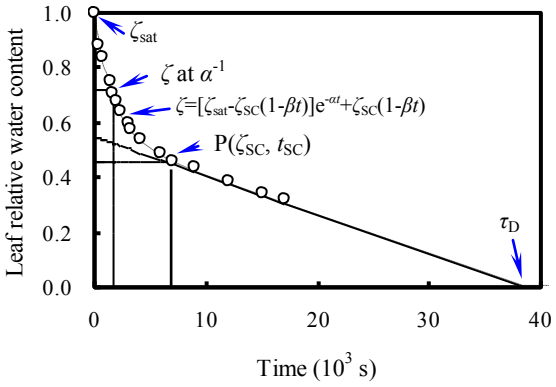

Fig. 3.1 A model of excised leaf transpiration declining curve.
τ_D is the time used to completely dry up the excised leaf; P (ζ_{SC}, t_{SC}), point at the zero turgor.

3.4 Results

3.4.1 AnM combined with mulching sent out of cotyledonary node and controlled the early flowering

In both mulched and un-mulched plots with AnM treatment, the hypocotyls elongated more than control (Fig. 3.2). The exposed hypocotyls changed the color from white through purple-red to green after the hypocotyls were exposed to light and low air humidity for several days. In the AnM plots irrespective of mulching, the seedling plants became a little shorter but thicker with leaves deeper in color, looking healthier compared with plants of control. The node of cotyledons was sent out of the surface of soil or the film mulch by the hypocotyls elongation in AnM plots while the cotyledonary node remained under the soil surface or film mulch in the plots without AnM treatment (Fig. 3.2). Two weeks after the hypocotyl exposure treatment, the plants grew better in MA plot of AnM combined with mulching than in MC plot without AnM treatment (Fig. 3.3). In the plots without AnM treatment, plants flowered earlier and some plants even flowered under the soil surface or the film mulch, while the early flowering in plots with AnM treatment was controlled by the hypocotyl exposure treatment. In both AnM and non-AnM plots, plants grow better in mulched treatments than in un-mulched treatments from the beginning throughout the whole growth period.

MC MA

Fig. 3.2 A peanut seedling with its hypocotyl elongated and exposed and the cotyledonary node sent out of soil surface in the modified AnM plot (MA) and a peanut seedling with its hypocotyl and cotyledonary node remaining under the soil surface in the MC plot. MA, the ridge was mulched by a bio-degradable black film with AnM practiced; MC, the ridge was mulched but AnM was not practiced.

A, MA B, NC C, MA D, MC

Fig. 3.3 Peanut plants grown with film mulched or AnM treatment. A, peanut plant grown in MA plot where the ridge was mulched by a bio-degradable black film with AnM practiced; B, peanut plant gown in NC plot where the ridge was not mulched and AnM was not practiced; C, plant gown in MA plot showed that the first node of peanut seedling is sent out of soil surface, and early flowering and seed setting are controlled; D, plant gown in MC (the ridge was mulched but AnM was not practice) plot showed that the first node of peanut seedling remains under soil surface, and flowering sets early.

3.4.2 Plant growth in the whole growth period was improved by AnM and/or mulching

As shown by the plant growth curves in Fig. 3.4, the dry mass per plant was a little lower at the beginning but burst higher in later growth period in AnM plots compared with those in plots without AnM treatment. The positive effect of AnM treatment on plant growth trend was similar to each other between mulched and un-mulched plots.

G_M, the maximum biomass increase, was higher in AnM than in non-AnM plot and much higher in mulched than in un-mulched plot with a significant synergistic interaction between mulching and AnM treatment (Table 3.3). β, the coefficient related with linear change by physical factors and related with slope of the sloping part of the plant growth curve, was a little higher in AnM than in non-AnM plots although the differences were small. Large value of coefficient α means high growth rate in the fast or the exponential growth stage. The AnM and/or mulching treatment increased plant growth shown by the high value of α. The time (day) that needed for the plant growth to reach half of the maximum shown by the constant τ, was 1 or 2 days earlier in AnM than in non-AnM plot and 10 days earlier in mulched than in un-mulched plot.

3.4.3 Photosynthetic activities were improved by AnM and film mulching treatment

AnM with mulching treatment (MA) improved the leaf photosynthesis by high photosynthetic capacity P_C, which AnM method without mulching (NA) and mulching only showed similar effect on leaf photosynthesis (Fig. 3.4 and Table 3.3). The respiration rate and the maximum quantum yield showed similar trends to P_C with synergistic interaction between AnM and mulching treatments. Chlorophyll content shown by the SPAD leaf color was increased by AnM and film mulching treatment. In overall, photosynthetic activities including photosynthetic capacity and the quantum use efficiency were improved by AnM and film mulching treatment.

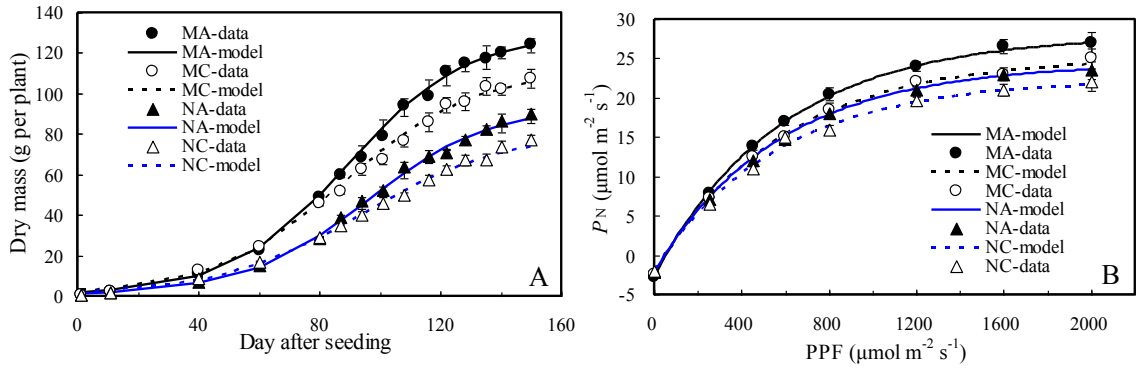

Fig. 3.4 Dynamics of plant growth (A) and photosynthetic light response curves (B) in peanut plants. MA, the ridge was mulched by a bio-degradable black film with AnM practiced; MC, the ridge was mulched but AnM was not practiced; NA, AnM was practiced without film mulching; and NC, neither AnM nor film mulching was practiced; data, the average of original data (n=12); model in A and B, the models analyzed by $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$ and $P_N = P_C(1-e^{-KI})-R_D$ according to the original data. Error bars represent SE values for twelve independent replicates.

Table 3.3 The growth analysis parameters, photosynthetic activities in peanut plants.

Treatment	G_M	G_B	β	α	τ	P_C	R_D	Y_Q	Color
	---(g pl^{-1})---		--(×10^{-3})--		(day)	--(μmol m^{-2} s^{-1})--		(mol mol^{-1})	(SPAD)
MA	129.1	0.91	3.36	48.2	94.5	30.7	2.7	0.0524	55
MC	111.1	0.91	3.28	42.3	96.5	27.6	2.4	0.0472	52
NA	93.3	0.91	3.59	44.1	104.4	26.6	2.2	0.0468	49
NC	80.2	0.91	3.36	38.4	105.7	24.4	2.0	0.0429	47
AnM	**	ns	*	**	*	*	*	**	**
Mulch	**	ns	*	**	**	*	*	*	**
A.×M.	*	ns	ns	ns	ns	ns	*	*	*

MA, mulching+AnM; MC, mulching without AnM; NA, AnM; and NC, control without AnM or mulching. P_C, photosynthetic capacity; R_D, respiration rate; Y_Q, maximum quantum yield.

The growth parameters from $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$. G_M, the maximum biomass increment; G_B, the original biomass of the plant at the beginning; α, the constant related with the steep part of the curve; β, the constant related with sloping part of the curve before and after the fast growth; τ, the time point when the biomass increment reached half amount of G_M.

*, ** and ns mean significance at $P\leq0.05$, at $P\leq0.01$ and no significance according to LSD analysis (n=12).

3.4.4 The final dry mass production and shell yield were improved by AnM combined mulching method

Both total plant and pod dry mass and the shell yield shown by both per plant and per square meter were significantly increased by AnM and mulching treatments with significant synergistic interaction between AnM and mulching treatment (Table 3.4). The yield increment by film mulching reached 47% and 44% in AnM and non-AnM plot, respectively. In the present experiment, an organic fertilizer was applied. The mulching might have promoted the mineralization of the organic materials and consequently increased the plant growth and yield. With chemical fertilizer, such a high yield increasing case has not been reported elsewhere. Even over so high increment, AnM treatment still increased shell yield by 19% in addition. The combined effect of AnM and film mulching on shell yield improvement reached as high as 71%.

3.4.5 AnM method prevented disease and rot shell

At the later growth stage, peanut leaves were infected by brown leaf spot (*Mycospaerella arachidis*). Both percentage of infected plants and disease index showed that the extent of infection were lowered by AnM and/or mulching treatments (Table 3.4). Rot shells, which might be contaminated by aflatoxin, were found in both mulched and un-mulched plots without AnM treatment but not found in AnM plot. This suggested that AnM technique prevented rotting of the early pods by controlling early flowering and early penetration of the pegs.

Table 3.4 Yield and disease index in peanut plants.

Treatment	Dry mass (g pl^{-1})		----Shell yield----		----Disease----		Rot shell
	Plant	Pod	(g pl^{-1})	(g m^{-2})	%	Index	(%)
MA	124.5	52.1	54.5	486.5	26.1	4.3	0.0
MC	107.6	43.5	46.1	409.1	34.6	8.6	1.3
NA	90.1	36.2	37.5	331.7	31.8	6.4	0.0
NC	77.7	31.3	32.2	284.1	39.7	12.8	1.1
AnM	**	**	**	**	*	*	**
Mulch	**	**	**	**	*	*	ns
A×M	*	*	*	*	*	*	ns

MA, mulching+AnM; MC, mulching; NA, AnM; and NC, control without AnM or mulching. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

3.4.6 AnM and mulching treatments improved water stress tolerance by osmotic adjustment

The leaf water potential the fully turgid status (Ψ_{FT}) was a little different between AnM and non-AnM plot but not between mulched and un-mulched plot (Table 3.5). The little difference might be caused by the difference in osmotic concentration in the cells and Ψ_{FT} was all in the un-stressful normal ranges. π_{FT}, the osmotic potential at full turgor, was lower in AnM plot than in non-AnM plot for both mulched and un-mulched peanut plants with a significant synergistic interaction. P_{FT}, the turgor potential at water-saturated condition, was calculated from the difference between Ψ_{FT} and π_{FT}. AnM treatment increased P_{FT} in both mulched and un-mulched peanut plants with a significant synergistic interaction. Leaf turgor potential is the driving force for plant to grow and for stomata to open in photosynthetic process. The improved leaf turgor potential was accountable for the improvements in photosynthetic activities and plant growth by AnM and film mulching treatments. Improved leaf turgor potential was the consequence of osmotic adjustment shown as the further lowered osmotic potential on the basis of leaf water potential similar to that in control (NC). C_{FT} was the osmotic concentration converted from the value of osmotic potential. Because exposing the hypocotyls to light and low air humidity was just a stimulus and did not cause real water stress, there was no cell water loss or the water loss-caused cell volume decrease. Therefore, there was no osmotic potential decrease caused by concentrating effect. Thus, the difference of osmotic potential or the osmotic concentration over the control value (ΔC_{FT}) showed the active increase in osmotic molecules caused by the treatments, which was defined as the osmotic adjustment ability (Turner 2003; Xu 2007). π_{s+a}, the osmotic potential in the symplastic solution theoretically diluted by apoplastic water, showed a similar trend to π_{FT}. π_{IP} was osmotic potential at incipient plasmolysis when the cell wall would separate from the symplasm because of the symplasm shrink. A lower π_{IP} would show a higher tolerance to cell dehydration. π_{IP} was lower in AnM than non-AnM plot and also lower in mulched than in un-mulched plot. With a similar meaning to π_{IP}, ζ_{IP} showed the leaf or cell relative water content at incipient plasmolysis. A lower ζ_{IP} showed a higher tolerance to cell dehydration. ζ_{IP} showed a similar trend to π_{IP}. Results of π_{IP} and ζ_{IP} suggested that AnM and mulching treatments improved water stress tolerance in peanut plants.

3.4.7 AnM and mulching treatment improved cell water condition

Water in a cell is actually compartmented in symplasm and apoplasm (Barlow 1986). It favorites biochemical functions and cell turgor maintenance if there is more fraction of water compartmented in symplasm especially in case of water deficit (Patakas and Noitsakis 1997). In the present experiment, it was found that ζ_{sym}, the symplastic water fraction, was larger, and ζ_{apo}, the apoplastic water fraction, was smaller in AnM than non-AnM plot and also lower in mulched than in un-mulched plot with a significant synergistic interaction between AnM and mulching treatment (Table 3.5). The constants

α and β were related with the shape of the P-V curve and reversely proportional to ζ_{IP} and ζ_{sym}, respectively. In overall, it was suggested that both AnM and mulching treatments improved cell water conditions for biochemical function in terms of cell water compartmentation.

3.4.8 AnM and mulching treatment improved midday leaf turgor potential

The midday leaf water potential (Ψ_{MD}) was slightly decreased by AnM but not by mulching treatment (Table 3.5). However, the leaf osmotic potential at midday (π_{MD}) was lower in AnM than non-AnM and also lower in mulched than in non-mulched plot. Consequently, the leaf turgor potential at midday (P_{MD}) was maintained higher in AnM than in non-AnM and higher in mulched than in non-mulched plot. The improvement in leaf turgor potential at midday would be accountable for the better plant growth, improved photosynthetic activities and high shell yield in AnM and mulched plots since the leaf turgor potential was the driving force for the plant to grow and for stomata to open in photosynthetic processes.

Table 3.5 The parameters from P-V curve analysis for peanut plants.

Treat.	Ψ_{FT}	Ψ_{MD}	π_{FT}	π_{MD}	P_{FT}	P_{MD}	π_{s+a}	π_{IP}	C_{FT}	ΔC_{FT}	ζ_{IP}	ζ_{sym}	ζ_{apo}	α	β
	----------(MPa)----------								-(osmol m^{-3})-						--($\times 10^{-3}$)--
MA	-0.23	-0.91	-1.59	-1.78	1.36	0.87	-1.34	-1.68	651.9	137.9	0.848	0.758	0.242	53.158	0.895
MC	-0.21	-0.90	-1.42	-1.60	1.21	0.70	-1.25	-1.53	582.2	68.2	0.870	0.724	0.276	50.177	0.964
NA	-0.22	-0.94	-1.39	-1.61	1.20	0.66	-1.20	-1.49	561.7	47.7	0.820	0.765	0.235	51.170	0.861
NC	-0.20	-0.92	-1.24	-1.45	1.07	0.52	-1.06	-1.0	500.2	0.0	0.880	0.731	0.269	45.531	0.875
Mulch	ns	ns	*	**	*	**	*	*	**	**	*	*	**	*	*
AnM	*	*	*	**	**	**	**	**	**	**	*	**	**	*	**
M×A	ns	ns	*	*	*	*	*	*	*	**	*	*	*	*	*

MA, mulching+AnM; MC, mulching; NA, AnM; and NC, control without AnM or mulching. The parameters were analyzed from P-V curve as follow:
$-\Psi^{-1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}])$. Ψ_{FT}, leaf water potential at the fully turgid status; Ψ_{MD}, leaf water potential at midday; π_{s+a}, the osmotic potential in the symplastic solution theoretically diluted by apoplastic water; π_{FT}, osmotic potential at the fully turgid status; π_{MD}, osmotic potential at midday; π_{IP}, osmotic potential at incipient plasmolysis; P_{FT}, turgor potential at the fully turgid status; P_{MD}; turgor potential at midday; C_{FT}, solute concentration at the fully turgid status; ΔC_{FT}, the difference in C_{FT} between the treatment and the control; ζ_{IP}, relative leaf water content at the incipient plasmolysis; ζ_{sym}, symplastic water fraction; ζ_{apo}; apoplastic water fraction; β, coefficient used to adjust the slope of the less sloping part of the curve; α, coefficient associated with the slope of steeply sloping part of the curve.
*, ** and ns mean significance at $P\leq 0.05$, at $P\leq 0.01$ and no significance according to LSD analysis (n=12).

3.4.9 AnM and mulching treatment increased leaf water retention ability

The leaf transpiration continued at the full speed for a while when a rehydrated excised leaf was placed under light with the cut trace placed in water. The leaf transpiration decreased in response to water deficit when the excised leaf was moved out from the water. Leaf water lost fast by stomatal transpiration at the beginning, which was quantitatively proportional to the value of α. The value of α was larger in leaf of peanut plant in AnM than in non-AnM plot and also larger in mulched than in un-mulched plot (Table 3.6). A larger value of α means a higher capacity of maintaining higher stomatal conductance for photosynthesis under leaf water deficit conditions. The maintenance of stomatal conductance is attributed to the leaf turgor maintenance. When the value of α is used to evaluate plant drought resistance, the mechanisms of drought tolerance and avoidance must be considered. For some plants with high drought tolerance, leaf turgor is maintained, stomatal transpiration is comparatively high and the speed of leaf water declining in the fast phase or the value of α is larger. The leaf relative water content (ζ_{SC}) at the stomata closure point was slightly decreased by AnM and mulching treatments. This suggested that peanut plants in AnM and mulched plots were more physiologically tolerant to leaf water deficit because they could open stomata to maintain photosynthesis under leaf water deficit conditions compared with those in non-AnM and un-mulched plots.

Actually, transpiration from the leaf does not stop even when the stomata are closed. The water loss slowed down caused by cuticular transpiration when stomata reached closing, which was proportional to the value of β. As usually known, when the cuticular layer is thicker or more waxes are deposited on or into the leaf surface, the transpiration after stomatal closure will be lower and β will be smaller. In the present experiment, the values of β were lower in AnM than in non-AnM plot and also lower in mulched than in un-mulched plot. This suggested that the leaves of peanut plants in AnM and in mulched plots might prevent water loss from the cuticular membrane by developing thicker cuticles and/or depositing more wax on the leaf surface, compared with the leaves in the corresponding control.

The constant τ_D represents the time when the leaf is completely dried or air dried to remain 10% of relative leaf water content. The value of τ_D was larger or the water retention duration was longer in leaves in AnM than in non-AnM plot and also larger in mulched than in un-mulched plot. In the field, peanut plants were healthier and stronger morphologically in AnM and mulched plots compared with the corresponding control. The improved morphology and cellular structure might also account for the abovementioned disease resistance.

Table 3.6 Parameters of the transpiration decline curve in the excised leave of peanut plants.

Treatment	ζ_{SC}	α	β	τ_D ($\times 10^3$ s)
MA	0.762	0.615	0.0088	97.9
MC	0.781	0.591	0.0104	84.4
NA	0.772	0.604	0.0095	90.9
NC	0.794	0.512	0.0113	76.8
Mulch	*	*	**	*
AnM	*	*	**	**
M×A	ns	*	*	*

MA, mulching + AnM; MC, mulching; NA, AnM; and NC, control without AnM or mulching. The parameters were analyzed from $\zeta=[\zeta_{sat}-\zeta_{SC}(1-\beta t)]e^{-\alpha t}+\zeta_{SC}(1-\beta t)$. ζ_{SC}, the leaf relative water content at the time when the stomata closed; β, the constant related with the slope of the second gently-sloping phase of the curve; α, the constant showing the slope of the first steeply-sloping phase of the curve; τ_D, the time used to completely dry up the excised leaf.

*, ** and ns mean significance at $P\leq 0.05$, at $P\leq 0.01$ and no significance according to LSD analysis (n=12).

3.5 Discussion

The AnM technique has been adopted in peanut production in China. The technique includes three links of practice, i.e., “A”, “n” and “M”. At “A” stage, seeds are sown a little deeper than usual into the ridge. The main objective of the “A” practice is to induce elongation of hypocotyl more than usual and to send the cotyledons out of the soil surface. When the cotyledons meet light, the hypocotyl stops elongating. Therefore, seeds must be sown a little deeper than usual in order to induce a longer hypocotyl. During the period of germination and hypocotyl elongation, the soil must be maintained moist enough to avoid crack of the soil surface. If the soil surface cracks, light would penetrate into the soil and stop the elongation of the hypocotyl. When the cotyledons are sent out of the soil surface, soils around the hypocotyl are removed away. This soil removal can be performed by a brush-like tail attached to a pulling machine, which can brush the soil away but does not damage the young seedlings. Because the practice meets a little difficulty, the modified AnM technique has been proposed and adopted in production. In this technique, the peanut seeds are sown 3 cm deep into the holes on the film mulched ridge and then the holes were covered with soil of 7 cm high. The covering soil is used to induce extra hypocotyl elongation. Because soil is covered onto the film, the ridge must be shallow or the seeds are just sown in flat bed with film mulched without ridge. The induced elongation of the hypocotyl, then in turn, sends the cotyledonary node out of the film surface. This is corresponding to the “A” stage of basic AnM. At the “n” stage, the soil is removed away when the cotyledonary node is sent out of the film surface. Unlike in the case of the basic AnM without film mulch, the soil in the ridge under the film surface is not removed any more. Two months after seeding, the soil in the furrows is earthed up to welcome the later pegs. This is the “M” stage treatment and similar to the case of basic AnM. It is easier to use machine to operate the modified AnM technique. Of course, the development and improvement of soil removing technique are left for the agricultural machinery specialists.

In agronomy, inducing elongation of the hypocotyl and exposing the hypocotyl are just used to send the cotyledonary node out of the soil surface and as far as possible away from the soil surface in order to control the early flowering and early penetrating of pegs. Exposure of the hypocotyls can send a signal to internal gene systems which delay the early flowering and peg elongation. Without “A” and “n” treatments, flowering would occur on the low cotyledonary node even inside the soil. The early fruit setting would send a signal to the internal gene systems, which make the plant to lose crisis sense and become lazy in seed setting process with the seed setting potential lowered and the final seed yield decreased. However, this is just speculations and there has been no molecular biological proof for these speculated signaling processes. Actually, the hypocotyl exposure is an artificial stimulus to induce xerophytophysiological regulations in addition to the abovementioned agronomical purposes. Usually, the hypocotyl grows under soil surface and does not meet light or

strong light and dry air. When exposed to light and dry air, the hypocotyl, especially the extra elongated one, perceives the unusual environmental changes and mistake artificial stimulus as a severe environmental stress. Actually, the peanut plants are supplied with sufficient water by the un-injured root system from the moist soil and there is no any real stress. However, the plant cannot be intelligent enough to distinguish the genuine of the stimulus. In the present experiment, it was observed in the field that the exposed hypocotyl changed its color from white through purple-red to green. The synthesis of pigments, probably anthocyanins, might be one of the consequences of xerophytophysiological regulations (Tahkokorpi 2010).

Osmotic adjustment in hypocotyl exposed peanut plants was confirmed in the present experiment. In response to the stimulus, the plant increased its solute concentration in the symplasm by keeping the small molecules not to change into large ones, breaking-down some large molecules into small ones, synthesizing new osmotically active molecules and uptaking more ions and solutes from the soil. The increased osmotic concentration helped to maintain a leaf turgor potential higher than usual, which in turn improved the leaf photosynthesis, plant growth and the final seed yield. One more xerophytophysiological regulation was the increased symplastic water fraction. For example, as in usual, water in a cell is compartmented 73.1% in the symplasm or inside the cell membrane and 26.9% in apoplasm or the cell wall. In response to the stimulus, the hypocotyl exposed peanut plants re-compartmented the cell water 76.5% in the symplasm and 23.3% in apoplasm. As suggested by Patakas and Noitsakis (1997), the 3.4% increase in symplastic water fraction might have favored the related biochemical metabolism and the improvement in cell turgor potential.

Another consequence of the xerophytophysiological regulations in peanut plants in the AnM methhod found in the present experiment was the improved leaf water retention ability, which might be caused by strengthened leaf surface morphological structure. The analysis showed that at the first phase during the excised leaf drying period leaves from AnM lost more water perhaps because of a higher stomatal conductance compared with the corresponding control. Soon the leaves closed their stomata and the water loss was almost only through cuticular transpiration. Leaves in AnM treatment closed the stomata at lower leaf water content than those from the control, as shown by the value of α, which was proportional to stomatal transpiration. This suggested that plants in AnM method were more tolerant to water deficit. This characteristic has been defined as drought tolerance as one component of drought resistance (Levitt 1980; Xu and Ishii 1996). In other words, a drought tolerant plant can sustain its physiological and biochemical performances despite of tissue dehydration. After stomata closed, peanut leaves in AnM plot lost leaf water less than those in control, as shown by the value of β, which was proportional to cuticular transpiration. As usual, cuticular transpiration would be lower if the cuticular layer was thicker or more wax was deposited into the leaf surface layer. Strengthened leaf surface structure

prevented water loss from cuticular transpiration when stomata closed especially under severe water deficit conditions. Usually, this characteristic is defined as drought avoidance. In other words, a drought avoidant plant can maintain its tissue water by avoiding tissue water loss through strengthened surface barriers (Coopman et al. 2008). Drought tolerance and avoidance in pair constitute the drought resistance. Even though there was no real drought and the consequent water stress in AnM treated peanut plants, the characteristics of drought tolerance and avoidance could make the plant healthier in the normal water conditions. The strengthened leaf surface structure might favor the disease resistance.

In similar ways, other stimuli have been used to induce xerophytophysiological regulations in many plants. The examples include mesocotyl exposure for sorghum plants (Xu et al. 2009d), clove exposure for garlic plants (Qin et al. 2008a), partial root-zone drying for tomato (Xu et al. 2009b) and potato (Xu et al. 2011a), restricted irrigation for house tomato (Xu et al. 2010), blue light irradiation in canopy for tomato crops (Xu et al. 2012), and seedling drying for transplanted wheat plants (Xu et al. 2011c). Even in hydroponic culture of crops such as tomato, moderate salinity can induce xerophytophysiological regulations. The expected xerophytophysiological regulations include increases in sugars, vitamin C, organic acids and other flavors, improved fruit color, strengthened disease and pest resistance and the improved final yield. In abovementioned applications of xerophytophysiology, crops are all grown under moderate water condition without real stresses, the stimuli are just used as signal starters to induce the expected regulations, and drought resistance, even if any, is not the objective. As reported by scientists at molecular biological levels, plants are intelligent to perceive changes in environmental conditions, such as drought characterized by high UV radiation, soil water deficit, low humidity and salinity and extreme temperature, and then to transduce the signals to the internals, which induce the related gene expression and activate metabolisms related with physiological and morphological regulations or adjustments in conferring stress tolerance (Jensen et al. 1996; Mulligan et al. 1997; Smith and Gallon 2001; Davis 2004). In most of the research cases, the model plant, *Arabidopsis*, is used and few crop plants have been involved in the molecular biological research (Walley and Dehesh 2010). Scientists have always in passive positions put emphases on breeding varieties of higher resistance to the adverse environmental conditions. However, the very benefits can be induced in an active position from xerophytophysiological mechanisms by giving plants a mild or even a false drought stimulus to make the plants healthier and stronger (Turner 1990). Actually, mulching is also a practice of xerophytophysiological applications, where condition of low humidity, mild dry soil and high soil temperature are created (Ramakrishna et al. 2006). In the whole Japan, watermelons are produced in a kind of small and low film tunnel with film mulched on the soil surface. The air humidity is low and temperature is high inside the tunnel but temperature gets down

much in the night. This creates environmental conditions similar to Turpan Basin, where sweet melons and grapes are produced. In the present experiment, the yield increasing effect by film mulching reached as high as more than 47%. Even on the so high basis of yield improvement by film mulching, AnM technique could still obtain a yield improvement of 19% and synergistic effect added to that of mulching reached as high as 71%. Therefore, results of the present experiment perfectly confirmed that practices of stimulation based on the theory of xerophytophysiology, such as the AnM technique, especially the modified AnM, were effective in inducing expected consequences of regulations in crop production. However, elucidation of the further detailed mechanisms is needed in the future research in molecular biological levels.

Chapter 4

Responses of Hypocotyl Elongation to Light and Sowing Depth in Peanut Seedlings

4.1 Abstract

Plant growth and health as well as the final shell yield is improved by AnM techniques including exposing the extra elongated hypocotyls to lift up the cotyledon node and induce xerophytophysiological regulations such as osmotic adjustment as described in the previous experiments. Elongation of hypocotyls must be ensured by the dark conditions for germinating seeds in soil, which needs the seeds being sown enough and properly deep. Therefore, in the present experiment, the effects of light and seeding depth on elongation and morphology as well as anthocyanins accumulation in hypocotyls of peanut seedlings were examined. A germinating hypocotyl of peanut stopped its elongation when the cotyledons met light even under the soil if the soil was cracked. The seeds should be sown deep enough, for example, up to 4 cm and 6 cm for cultivars 'Chibahandachi' and 'Nakateyutaka' respectively. Light strongly induced anthocyanin accumulation as observed in purple-red hypocotyls of peanut seedlings. The light effects of elongation inhibition, changes in tissue and cell morphology and anthocyanin accumulation in hypocotyls were stronger in cultivar 'Nakateyutaka' than in 'Chibahandachi'. Although the photosynthetic functions were induced by light, the short hypocotyl and early development of the cotyledon node branches were not appreciated agronomically because, as described in our previous experiments, the early development of cotyledon node branches may make many problems for the later growth of the peanut crop. An extra elongated hypocotyl is also needed for the treatment of hypocotyl exposure to induce xerophytophysiological regulations.

Key words: AnM technique, anthocyanin, histological and anatomical observation, hypocotyl elongation, light, peanut (*Arachis hypogaea*), sowing depth.

4.2 Introduction

Peanut (*Arachis hypogaea* L.) is an unusual species in legume family with its flowers borne in air but developing its pods underground. Peanut yield is limited by the characteristic of cotyledonary node remaining inside soil and early seeding that negatively affects the later plant growth and final shell yield by nutrition competition and some kind of signaling to show satisfactory of having seeds for next generation (Shen and An 1988; Baldet et al. 2006). As the peanut seedling grows from germination, elongation of hypocotyl lifts the cotyledons and young epicotyl above the ground, giving rise to the first true leaves and then the first two branched from the cotyledon node. However, not like soybean or other legume plants, the cotyledons of peanut do not or do not completely emerge from the soil in most cases because the hypocotyl elongation stops when the cotyledons meet light even under the soil when the light penetrates from the cracks (Shen 1985) . Even in case of emergence from the soil, the cotyledonary node may remain inside the soil. Branches from the cotyledonary node and the early flower buds are usually covered by soils. These underground pollinated pods ripe early and rot inside the soil, resulting in aflatoxin contamination of the product (Shen 1990). The early pods compete with the whole plant and the later flowers for nutrition. The early seed setting may send a signal to the plant internal gene system to show that the plant has already seeds for the next generation and no much effort needed to make more seeds. Therefore, the final shell yield may be decreased by the early seed setting. AnM method is brought up based on the specific feature of peanut that hypocotyl elongation stops when cotyledons expose to light (Shen and An 1988). Seeds are usually sown in a ridge a little deeper (8 cm) than normal to induce the hypocotyl to elongate to an enough size. Deep sowing enables the hypocotyl to elongate enough, and then the topsoil is removed away, the hypocotyl is exposed to light and dry air and the cotyledonary node is completely aboveground. Elongation of hypocotyl needs much time or even the cotyledons remain inside the soil and never come out before emergence of the cotyledons if seeds are sowing over deeply. If seeds are sown too shallowly beneath the surface soil, hypocotyls stop to elongate and grow very short because cotyledon meets light too early. In this case, light is the main limiting factor for hypocotyl elongation. In AnM techniques, exposure of the extra elongated hypocotyls to light and dry air is also an artificial stimulation to induce xerophytophysiological regulations such as osmotic adjustment improving leaf turgor and surface structure strengthening ensuring the plant healthy (Xu 2007). Therefore, the present experiment is designed for three purposes, 1) to confirm the optimal sowing depth in AnM method; 2) to analyze the dynamic hypocotyl elongation; and 3) to examine the effects of light and depth on hypocotyl growth including histological analysis.

4.3 Materials and Methods

4.3.1 Plant materials and planting conditions

Seeds of peanut (*Arachis hypogaea* L. cv. Chibahandachi and Nakateyutaka) were selected in an even size shown by the seed biomass (0.88 g seed^{-1} in average) and sown in transparent round plastic bottles of 0.5 L filled with the substrate of humus soil combined with sandy loam (v:v = 3:1). Six seeds were symmetrically arranged in each bottle along the side wall of the bottle so that the elongation of hypocotyls can be clearly observed visually and germinating seeds can be exposed to sunlight through the transparent wall in the following mentioned light treatment. The experiment was performed in a polyethylene film-covered greenhouse under natural conditions with ambient light and temperature, with the day/night temperature as 28±0.5/20±0.5˚C and the relative humidity as 60±10% during the experiments.

4.3.2 Treatments

The experiment was conducted as a random 2×2 factorial design with the light and the sowing depth as the horizontal factors. Four sowing depth treatments were 2, 4, 6 and 8 cm from the top of the soil surface, with six seeds symmetrically sown in each bottle. Half of bottles containing seeds in four sowing depths were placed in a dark as control enclosed by black plastic covers, while the other half of sowing-depth-treated bottles remained exposed to natural light. The lighting time was according to the variations of diurnal light period. Both light and sowing depth treatments were replicated 3 times.

4.3.3 Analysis of hypocotyl elongation

The length of hypocotyls was measured every day after the hypocotyls began to elongate. Measurements were ended when the elongation of the hypocotyl stopped. The dynamic elongation of hypocotyls was analyzed by the growth dynamics curve equation modified according to Qin et al. (2008b), $l_H = l_T\,[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}+l_o(1-\beta t)$.

Here, l_T is the total increment in length by growth; l_o is the length at the time when hypocotyls begin to elongate; α is a constant associated with the fast growth; β is a constant of other factors except the growth factors; τ is the time point for the length increase to reach the half maximum; and t is the growth time (day).

4.3.4 Anatomic observation of hypocotyls

Samples of hypocotyls were prepared by standard free-hand sectioning method (Ruzin 1999; Lux et al. 2005). The sections were cut transversely and longitudinally with smooth strokes and transferred from the blade into a drop of water on a microscope slide. The well-prepared sections were then observed under optical and fluorescence microscopy (Olympus, Tokyo, Japan) equipped with an air-cooled CCD camera VB-7010 (KEYENCE, Osaka, Japan) and VB-Viewer software (KEYENCE).

4.3.5 Anthocyanin measurement

The surface tissue 1-2 mm thick was peeled from the part of the swollen hypocotyl tissue and trimmed to a 1×1 cm section. The peeled tissue sections were soaked in 0.5-3

ml of methanol acidified with 1% HCl in dark for 18 h at 4˚C. Three sections from each seedling were combined for each measurement. The absorbance of the extract at 530 nm was measured by a spectrophotometer (Hitachi U-2000, Tokyo, Japan) after the extract was diluted with 1% HCl acidified methanol depending on the anthocyanins concentration. The relative concentration of anthocyanins was expressed as optical density (OD_{530}) per cm^2 of hypocotyl tissue (Zhou et al. 2007; 2008).

4.4 Results

4.4.1 Seeding depths were 4 cm and 6 cm for enough elongation of hypocotyls in the cultivars 'Chibahandachi' and 'Nakateyutaka' respectively

Agronomically, one of the specific characteristics for the peanut plant is that the hypocotyl stops elongation when the cotyledons meet light. If the surface soil cracked because of drought and then the light penetrated into the soil, the hypocotyl would stop its elongation and make the cotyledons remaining inside the soil. The base part of the first two branches from the cotyledon node remains inside the soil, where early flowers are self-pollinated and pods form earlier than usual competing for nutrition with the young plant (Shen 1985). The early pods ripen too early before harvest and rot away, are infected by *Aaspergillus flavus* and produce aflatoxin, which in turn contaminates the shell product (Shen and An 1988; Cole 1989; Shen 1990). One of the agronomic purposes of AnM techniques is to solve this problem by inducing hypocotyl elongation to lift up the cotyledon node. Therefore, the proper seeding depth is the first for the AnM techniques.

In the present experiment, the shape of elongation curve is sigmoid like the italic letter "*S*" exhibiting three phases, the initially steady sloping part, the sharply-sloping part and the last steady sloping part. Under dark, hypocotyl of cultivar 'Chibahandachi' grew at the depth of 4 cm showed different elongation curve from those at other three depths (Fig. 4.1A), and elongated to 6.23 cm (l_T) (Table 4.1). The maximum length increment (l_T) at 2, 6 and 8 depth treatments was less than 5 cm. As shown by the value of τ. the time point for the length increase to reach the half maximum, was 7.12 day at the depth of 4 cm. When seedlings grew at the depth of 8 cm, it took more days for hypocotyl elongation and even seedlings did not come out of the soil surface. This probably caused by the too deep of the soil. In cultivar 'Nakateyutaka', the elongation curves in D-6 and D-8 treatments were different from those in D-2 and D-4 treatments (Fig. 4.1 C). The l_T was 4.26 and 5.01 cm in D-6 and D-8 treatments, respectively, higher than those in D-2 and D-4 treatments where l_T was only 3.29 and 3.62 cm, respectively (Table 4.2). The hypocotyl of cultivar 'Nakateyutaka' elongated more if the seed was sown deeper up to 8 cm while the best seeding depth was considered as 6 cm for hypocotyl elongation because at the depth of 8 cm, seedlings grew much longer epicotyl caused the too deep soil, which negatively affected the later growth. In addition, it took only 4.85 day (τ) for hypocotyl to elongate to the half maximum, compared with more than 6 days at the depth of 2 and 4 cm. Moreover, it took only 9 days for 'Nakateyutaka' to emergence while as long as 15 days were needed for 'Chibahandachi'. Therefore, the seeds should be sown deep enough, for example, up to 4 cm and 6 cm for enough elongation of hypocotyls in the cultivars 'Chibahandachi' and 'Nakateyutaka' respectively, if the AnM method was used.

4.4.2 Effect of lighting and cultivar difference.

Light significantly depressed hypocotyl elongation, with the maximum length under

most light treatments in the range of only 1.0-1.5 cm (l_T) in the cultivars of 'Chibahandachi' and 'Nakateyutaka' (Fig. 4.1 B, D; Tables 4.1 and 4.2). Seedlings still possessed the ability to emerge from soil under light condition, i.e., from 4-cm deep soil in 'Chibahandachi' and from 6 and 8-cm in 'Nakateyutaka'. The cotyledons inside the soil met the light from the crack, making the hypocotyl to stop its elongation, with the cotyledon node and part of the cotyledons remaining in the soil. Light inhibition effect on hypocotyl elongation was higher in cultivar 'Nakateyutaka', of which the ability of hypocotyl elongation was about 4 cm, shorter than in 'Chibahandachi'.

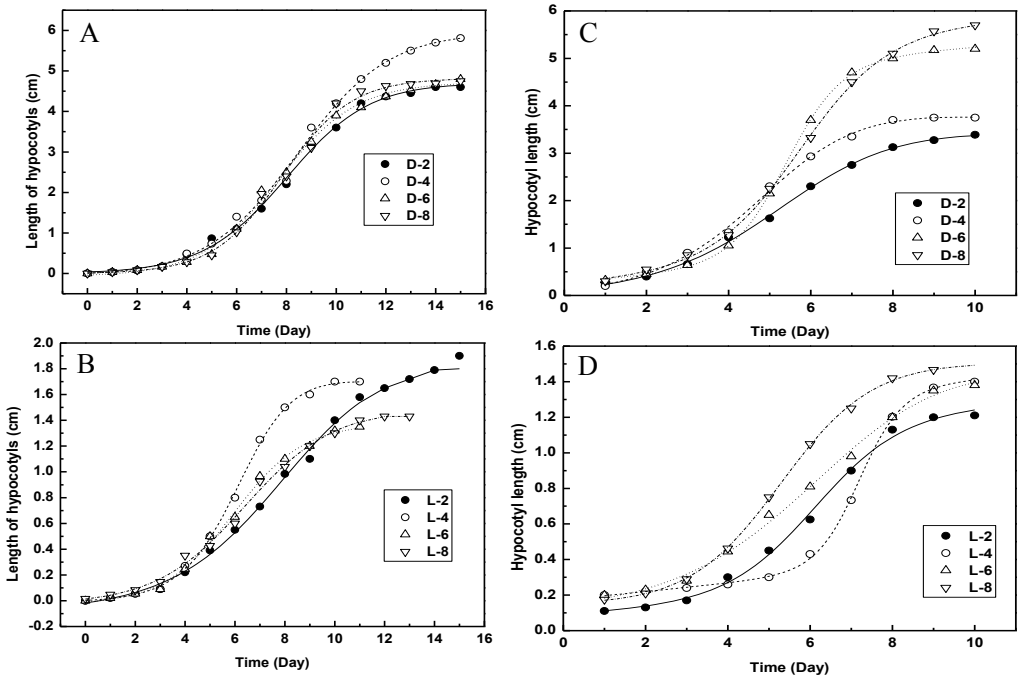

Fig. 4.1 Effect of sowing depth on elongation dynamic of hypocotyls in peanut seedlings. A, 'Chibahandachi' grew in dark; B, 'Chibahandachi' grew in light. C, 'Nakateyutaka' grew in dark; D, 'Nakateyutaka' grew in light. D-2, D-4, D-6 and D-8 were sowing depths at 2, 4, 6 and 8 cm, respectively, in dark; L-2, L-4, L-6 and L-8 were sowing depths at 2, 4, 6 and 8 cm, respectively, in light condition. Data represent average for three independent replicates.

Table 4.1 Parameters of dynamic elongation curves of hypocotyls in peanut 'Chibahandachi' seedlings sown at the depth of 2, 4, 6 and 8 cm from the surface soil under dark or light condition.

Depth	----l_T (cm)----		-----l_o (cm)-----		------α------		------β------		---τ (day)---	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light
2	4.88	2.05	-0.033	-0.082	0.67	0.45	-0.275	-0.018	6.24	7.60
4	6.23	1.67	-0.084	-0.010	0.60	0.66	-0.139	0.104	7.12	7.45
6	4.80	1.41	-0.074	-0.031	0.64	0.72	0.002	-0.011	7.72	5.84
8	4.50	1.46	-0.066	-0.031	0.46	0.46	0.090	0.068	10.24	7.49
Depth	*	*	*	**	*	*	**	**	**	*
Light	**		**		*		**		*	
Depth×Light	*		*		*		**		*	

Parameters were analyzed by the growth dynamics curve as follow,
$l_H = l_T\ [1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}+l_o(1-\beta t)$. l_T, the total increment in length by growth; l_o, the length at the time when hypocotyls begin to elongate; α, a constant associated with the fast growth; β, a constant of other factors except the growth factors; τ, the time point for the length increase to reach the half maximum (day). ns, * and **, non-significance, significance at $P\leq0.05$ and $P\leq0.01$, respectively, according to LSD analysis.

Table 4.2 Parameters of dynamic elongation curves of hypocotyls in peanut 'Nakateyutaka' seedlings sown at the depth of 2, 4, 6 and 8 cm from the surface soil under dark or light condition.

Depth	----l_T (cm)----		-----l_o (cm)-----		------α------		------β-----		--τ (day^{-1})--	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light
2	3.29	1.05	0.028	0.089	0.46	1.01	0.112	-0.150	6.62	5.47
4	3.62	1.01	0.027	0.173	0.54	2.00	0.129	-0.137	6.07	6.85
6	4.26	1.35	0.276	0.081	1.58	0.36	-0.254	0.107	4.85	7.97
8	5.01	1.42	0.230	0.144	1.02	0.84	-0.237	0.035	4.88	5.53
Depth	**	**	**	*	**	**	**	**	**	**
Light	**		**		**		**		*	
Depth×Light	*		*		**		**		*	

Parameters were analyzed by the growth dynamics curve as follow,
$l_H = l_T\ [1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}+l_o(1-\beta t)$. l_T, the total increment in length by growth; l_o, the length at the time when hypocotyls begin to elongate; α, a constant associated with the fast growth; β, a constant of other factors except the growth factors; τ, the time point for the length increase to reach the half maximum (day). ns, * and **, non-significance, significance at $P\leq0.05$ and $P\leq0.01$, respectively, according to LSD analysis.

4.4.3 Light induced accumulation of anthocyanins in hypocotyl

In addition to inhibiting hypocotyl elongation, light strongly induced the red-purple pigmentation attributed to anthocyanin accumulation in hypocotyls of peanut seedlings. Fig. 4.2 showed the anthocyanin concentration in hypocotyl of two peanut cultivars on the day when hypocotyls stopped elongation or cotyledons met light. Anthocyanin accumulated to higher concentration in hypocotyls of 'Nakateyutaka' than in 'Chibahandachi', with the exception in L-6 and L-8 treatments where the deep soil might block part of solar radiation to hypocotyls (Fig. 4.2 B). When seeding depth was low, the cotyledons met light earlier and higher anthocyanin concentration was detected especially in 'Nakateyutaka'.

Anthocyanin started to accumulate from the initial stage after seeds sown. Fig. 4.3 showed the anthocyanin accumulation at three growth stages. As hypocotyls grew to the fast elongation stage (the sharp-sloped phase shown by dynamic curve), anthocyanin concentration kept on accumulating to a high level and reached the maximum at/before the seedling emergence. Some seedlings grew with green hypocotyls because the biosynthesis of photosynthetically competent chloroplasts induced by long time of light irradiation overlayed the pigmentation of anthocyanins (data not shown). In 'Chibahandachi', anthocyanin concentration induced by light in hypocotyl showed no different among the sowing depth, with the exception of 2 and 4 cm depth at day 5, and the anthocyanin was higher than other two depth treatment (Fig. 4.3 A). Higher concentrations of anthocyanin in 'Nakateyutaka' were induced by light 7 days after seeds were sown in the depths of 2 and 4 cm than at other seeding depth treatments (Fig. 4.3 B). Deep seeding inhibited anthocyanin accumulation. There were also large differences in anthocyanin accumulation between the two peanut cultivars (Figs. 4.2, 4.3). In overall, higher concentration of anthocyanins was induced by light in cultivar 'Nakateyutaka' than in 'Chibahandachi'.

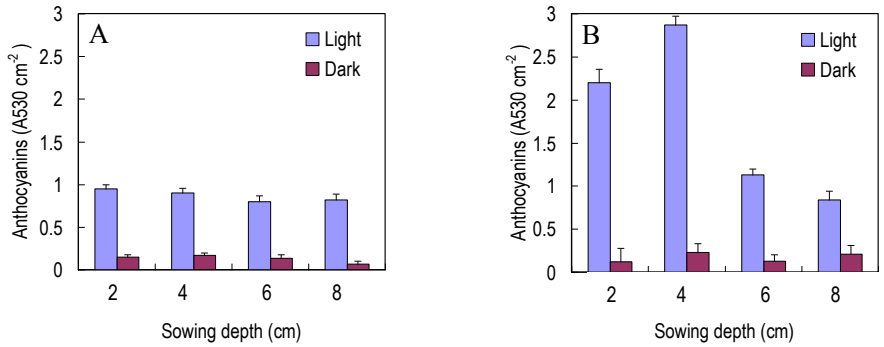

Fig. 4.2 Anthocyanin accumulation in hypocotyls of peanut seedlings with different sowing depth at day 13 after seeding. A, 'Chibahandachi'; B, 'Nakateyutaka'. Error bars represent SE values for three independent replicates.

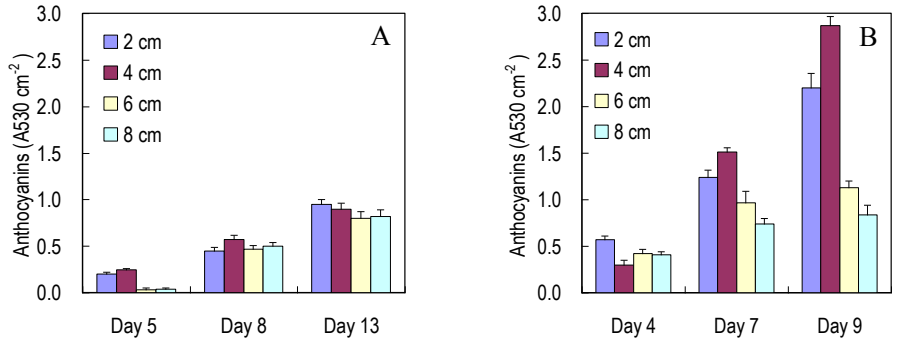

Fig. 4.3 Anthocyanin accumulation in hypocotyls of peanut seedlings grown under light at different stages. A, 'Chibahandachi'; B, 'Nakateyutaka'. Error bars represent SE values for three independent replicates.

4.4.4 Hypocotyl cell morphology

Under dark conditions, hypocotyls elongated enough to lift up the cotyledon nodes out of the soil when seeds were sown 4 and 6 cm deep in 'Chibahandachi' and 'Nakateyutaka', respectively, before cotyledon emerged from soil being exposed to sunlight (Tables 4.1 and 4.2). Morphologically, hypocotyls of both cultivars under light condition grew short but swollen with unfolded cotyledons both containing pigments and photosynthetically competent chloroplasts.

Hypocotyls of both cultivars grown in dark showed extensively elongated cells, rectangular in shape (Fig. 4.4 A), whereas the light-grown hypocotyl cells were only enlarged, oval-shaped and closely packed along the vertical axis (Fig. 4.4 B). These closely packed cells were extruded like flattened half-elliptic balls as result of inhibited elongation.

Epidermis of hypocotyls was stripped and used to investigate the effect of light on morphology of epidermal cells. Epidermis of dark-grown hypocotyls was constructed of large and rectangular-shaped cells (Fig. 4.5 A). Morphologically, hypocotyls showed a smooth surface. In light-grown hypocotyls, epidermal cells were small and polygonal compared with those under dark condition, indicating that long period grown in the light led to more cell enlargement and cell division (Fig. 4.5 B). The ability of these irregular and closely packed cells was suppressed to elongate but only swallowed, giving rise to a ridged surface of hypocotyls (Fig. 4.5 E).

The mature stomatal structures appeared in the epidermal cells of light-grown hypocotyls (Fig. 4.5 B-F). Most of stomata initiated and developed in the slightly ridges of hypocotyl surface (Fig. 4.5 E). Hypocotyls grown under long period of light changed histological development of their epidermal cells in adoption to unexpectedly adverse environment. The stomata formation in the hypocotyl surface suggested that the epidermal cells possessed the photosynthesis machinery that could not occur in dark-grown hypocotyls.

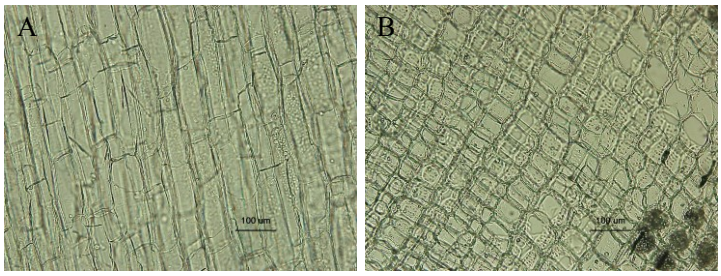

Fig. 4.4 Longitudinal sections of cortex in hypocotyls of thirteen-day-old 'Chibahandachi' seedlings observed under white microscope. A, under dark; B, under light. Bar = 100 μm.

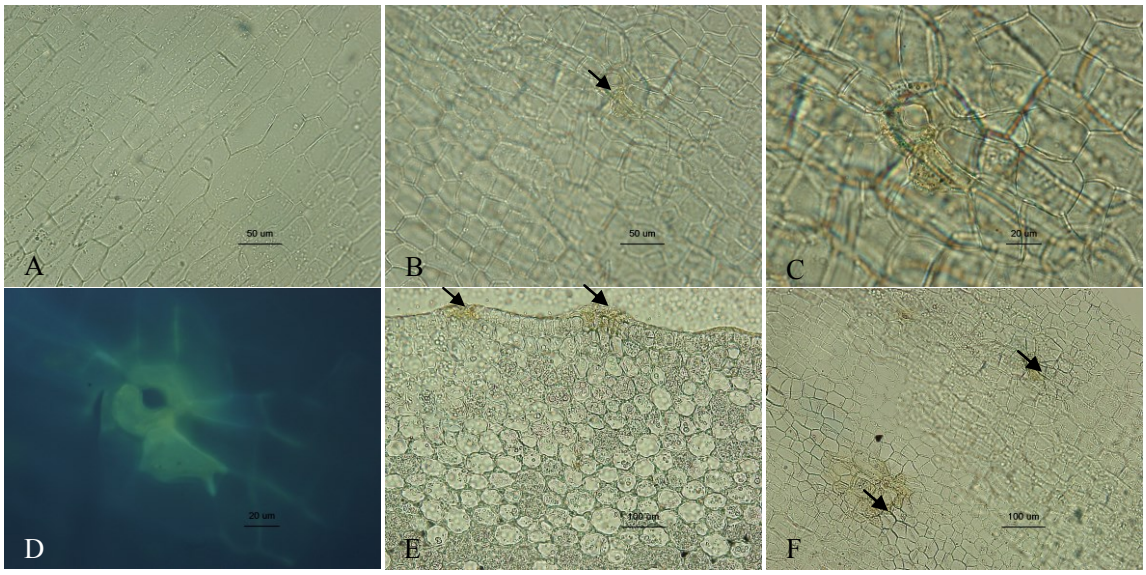

Fig. 4.5 Epidermal and cortex cells as well as initiation or maturation of stomata (C-F) in epidermis of light grown hypocotyls. A, epidermal cells from hypocotyl under dark, ; B, epidermal cells from hypocotyl under light; C, D and F, Stamata from epidermal cells of light grown hypocotyl observed under white microscope and UV light; E, cross section from light grown hypocotyls. A and B, Bar = 50 μm; C and D, Bar = 20 μm; E and F, Bar = 100 μm. Arrowheads represented stomata.

4.5 Discussion

In China, peanut seeds are sown on a small ridge without any other cultivation treatment during their whole growth period in the traditional method in peanut production. However, AnM method is a cultivation method according to the specific biological characteristics in different developmental stages in peanut plant. The whole growth period in peanut plant can be divided into three important stages at which time three cultivation treatments are made by managing the ridge shape. The first treatment is deep sowing at seed sowing time. The second treatment is hypocotyl exposure at seedling stage by removing the soil away around with hypocotyl and part of the roots exposed. And the last treatment is earthing up soil to welcome the pegs at the full blossom stage.

The present study focused on the first "A" stage. The main point of "A" stage lies in the fact that peanut cotyledons do not or do not completely emerge from the soil because hypocotyl stops to elongate once cotyledons meet light (Angelo 1973; Hoshikawa 1980; Shen and An 1988; Shen 1990; Ramanatha and Murty 1994; Wan 2003). The deep sowing treatment can induce hypocotyl to elongate enough and then the top soil is removed, and the two cotyledons are completely emerged. The "A" stage treatment is a preparation for the next treatment at "n" stage when the soil is removed away around seedlings with the entire hypocotyls and the basal part of the root exposed. At this time, if the hypocotyl is enough long, the base of the seedlings, especially the branches, is far enough from the soil surface. Peanut develop their flowers from the lower to upper parts of their plant bodies. Therefore, the early pegs from the early flowers penetrate into soil earlier followed by the pegs from the later flowers in the upper part of the plant. With AnM method, it will take comparatively long time for the early pegs from the early flowers to penetrate into soil. While, the early pegs in the plants without AnM method form underground or penetrate into soil very soon after they formed because hypocotyls do not elongate enough before cotyledons meet light. The short hypocotyls fail to push the cotyledons completely up through the soil and the basal parts of the two main branches from the cotyledonary nodes remain buried by the soil. In most cases, the early flowers from the base of the branches form underground or just above the soil surface. That's why to induce an enough long hypocotyl is the first step for the AnM method. Hypocotyl grows at the range of 1-11 cm according to varieties and mainly depends on the depth of planting (McDonald et al. 1987; Ramanatha and Murty 1994). In the present study, the enough length of two Japanese peanut cultivars, 'Chibahandachi' and 'Nakateyutaka', was confirmed at the depth of 4 cm and 6 cm, respectively, before AnM method was conducted.

The modified AnM method, a method of plastic mulch combined with AnM method, shows its more advantages in inducing the emergence of cotyledon internodes (Shen and An 1988; Shen et al. 1996). Plastic mulch with 5 cm high heap of soil covered above postpones the light shining upon seedlings and the cotyledons can come out by

themselves 0.74 cm higher above the surface of mulch, avoiding cotyledonary nodes inside soil (Shen et al. 1996). Moreover, if hypocotyls grow too short after exposure to light but less than 5 days, the stop-elongated hypocotyls can return to elongate by raising the thickness of the above heap of soil again. This measure can successfully lead the cotyledonary nodes out of soil, consequently making the seedlings hardened (Shen et al. 1996).

There have been many reports on light-induced inhibition of hypocotyl elongation in plants. Hypocotyl is a part of a germinating seedling of a plant in most dicot species. As the plant embryo grows at germination, it sends out downward the radicle, which becomes the primary root and penetrates into the soil, and lifts the growing tip (usually including the seed coat) above the ground, bearing the embryonic leaves (called cotyledons) and the plumule that gives rise to the first true leaves (from Wikipedia, the free encyclopedia). A dicotyledon germinating in dark develops an etiolated seedling with a rapidly elongating hypocotyl and unopened cotyledons containing no photosynthetically competent chloroplasts. Under etiolation development mode, most of the plant seedlings are channeled into elongation of the hypocotyl (Lin 2002). The elongating hypocotyl sends out the cotyledons to emerge rapidly from soil. In contrast, seedlings grown in the light follow a different path, including the inhibition of etiolation and initiation of the greening process that enables light capture through photosynthesis which are called de-etiolation or photomorphogenesis (Fankhauser and Chory 1997; Lin 2002; Vandenbussche et al. 2005; Nozue and Maloof 2006). The de-etiolation responses include inhibition of hypocotyl elongation, stimulation of cotyledon opening, cellular and subcellular differentiation, organ morphology, and the induction of pigments biosynthesis and gene expression (Fankhauser and Chory 1997; Lin 2002).

Rapid inhibition of hypocotyl elongation by light, including natural light, blue/UV light (Ahmad et al. 1995; Folta and Spalding 2001; Chen et al. 2004), white light, and red/far-red light (Wildermann et al. 1978; Hennig et al. 1999), has been studied extensively in the last three decades in seedlings of many dicotyledonous species. In the present study, seedlings were kept in 24 hrs of dark and 14/10 hrs of light/dark. In the light/dark condition, seedlings were followed in the diurnal cycles of light and dark so that cotyledons can be exposed to light. We found the strong inhibition of cell extension, the initiation and maturation of stomata in the epidermal layer and the accumulation of anthocyanins in light-grown hypocotyls of peanut seedlings. Usually, there was impossible for stomata development in organs like root and hypocotyls that should have grown underground. The initiation and maturation of stomata in light-grown hypocotyls implied that the long period exposure to light induced the developmental changes of photomorphogenesis so that hypocotyls could be adapted to the light growing condition (Wei et al. 1994). Moreover, the biosynthesis of anthocyanins and the initiation of the greening process enable light capture through photosynthesis. In the present study, the

peanut seedlings were not kept in continuous light but kept in diurnal condition. The integrated study by Nozue et al. (2007) has explained hypocotyl growth under diurnal cycles of light and dark. They noticed that seedlings with specific defects in light perception had weak or no growth rhythms, suggesting that light signaling is essential for rhythmic growth under diurnal cycles. And they also showed that seedlings seemed to tune their maximum growth at dawn (Breton and Kay 2007; Nozeu et al. 2007).

An accompanying phenomenon with inhibition of hypocotyl elongation is light-induced anthocyanin biosynthesis. Anthocyanins, as water-soluble pigments, may be environmentally transient, appearing and disappearing with changes in photoperiod, temperature, soil moisture or other signals (Chalker-Scott 2002). In the present study, anthocyanins accumulated to the maximum through three growth stages, the initial steady growth stage, the sharp-sloped stage and the final growth stop stage, by about two weeks diurnal light. Light is considered as one kind of environmental stress to the developing seedlings as dark is necessary for seed germination in addition to oxygen and water. The response of anthocyanin accumulation may provide a protective mechanism either against light-induced photooxidation or against damaging levels of light, especially high-energy blue light or UV irradiations (Drumm-Herrel 1984; Scott 1999; Stone et al. 2001; Steyn et al. 2002; Close and Beadle 2003; Guo et al. 2008; Hatier and Gould 2008). For young tissues which lack adequate structural protection to avoid photooxidation, anthocyanins can significantly attenuate high radiation (Scott 1999). As anthocyanins are extremely soluble in water, it is believed to be osmotic adjustors in avoidance to the osmotic stress induced by adverse environments such as light, drought, heat, cold, wind, flooding and saline conditions (Chalker-Scott L. 1999; Chalker-Scott 2002; Nagira et al. 2006). Anthocyanins may serve to decrease leaf osmotic potential, which could increase water uptake and/or reduce transpirational losses and allow anthocyanin-containing leaves to tolerate suboptimal water levels (Choinski and Johnson 1993; Chalker-Scott 2002; Tahkokorpi 2010). Plant tissues containing anthocyanins are often resistant to drought stress although the drought resistance is not causatively linked to anthocyanin content (Paine et al. 1992; Sherwin and Farrant 1998; Scott 1999).

In the present research on AnM techniques in peanut production, artificial stimulation is imposed onto the seedlings by exposing the hypocotyl elongated to induce expected positive regulations based on xerophytophysiology (Xu 2007). Accumulation of anthocyanins, which can be visually observed in the experimental peanut seedlings, could also be considered as one of consequences of xerophytophysiological responses induced by AnM techniques. Plant tissues containing anthocyanins are usually more resistant to drought and other stresses (Chalker-Scott 2002). The presence of anthocyanins is also an indication of plant health if no stresses are present as in case of my experiments on AnM techniques, where peanut seedlings are always maintained in optimum soil moisture conditions. Accumulation of

anthocyanin induced by light has also been found in hypocotyls of many other plants such as garden balsam (Arnord and Alston 1961), buckwheat (Troyer 1964), eggplant (Toguri et al. 1993) and turnip (Zhou et al. 2007; 2008), where anthocyanins play positive roles in making a healthy crop.

In conclusions, a germinating hypocotyl of peanut would stop its elongation when the cotyledons met light even under the cracked soil. Light significantly inhibited the elongation of hypocotyl by inhibiting the elongation of the individual cells. A sufficiently extra elongated hypocotyl is needed for the treatment of hypocotyl exposure in AnM method. Therefore, a seed of peanut needs to be sown enough and properly deep to induce the elongation of hypocotyl. In the present experiment, it was found that seeds should be sown deep enough, for example, up to 4 cm and 6 cm for cultivars ‘Chibahandachi’ and ‘Nakateyutaka’ respectively. Anthocyanins in the surface cells of hypocotyl induced by light would protect the photosynthetic functions of young chloroplasts.

Chapter 5

Modified AnM Technique in Combination with Black and Transparent Film Mulching in Peanut Production

5.1 Abstract

The modified AnM through exposing the extra elongated hypocotyls in combination with film mulching has proved effective in improvements in plant growth and shell yield by inducing xerophytophysiological regulations. In the present experiment, black film and white transparent film were compared in the modified AnM technique in the peanut crop. Results showed that black film and white film were equally effective in improvements in plant growth, photosynthesis, and the final shell yield if the AnM practice was adopted. As shown by pressure-volume curve analysis, inducing the hypocotyl elongation and exposing it to light and dry air induced osmotic adjustment and the consequent increases in leaf turgor and the symplastic water fraction. This result was confirmed by the direct measurements of the solutes such as sugars and proline, which were increased by hypocotyls exposure, as well as the protein, which was instead decreased. Excised leaf transpiration declining curve analysis suggested that exposing hypocotyls decreased cuticular transpiration.

Keywords: AnM technique, drought, osmotic adjustment, peanut (*Arachis hypogaea* L.), signal transduction, xerophytophysiology.

5.2 Introduction

Most cultivars of peanut crops grown in Japan and China belong to *Arachis hypogaea*, the Virginia type. As the same as most of the legumes, during the germination of a peanut seed, the hypocotyl first elongates in the soil and sends the cotyledons out of the soil surface. In many cases, the cotyledons meet light and the hypocotyls stop elongation when the light penetrates into the soil and irradiate the cotyledons because of soil surface cracking, especially in drought conditions. In these cases, cotyledons remain under soil surface and the first pair of branches develop from the cotyledon node and suffer the adverse conditions such as low light and high humidity under soil surface. The early branches develop their flowers, which pollinate by themselves, develop pods and sets seeds earlier than usual. The early pods have several disadvantages: 1) using up the carbohydrates produced by the young plant that has not developed enough and so inhibiting the plant development by nutrition competition; 2) inducing a signal to inform the plant that it has already had offspring and making the plant to be lazy or lose its crisis sense in further seeds producing; and 3) the ripen early pods would rot in soil, infected by fungi that producing aflatoxin, which in turn contaminates other pods (Shen 1958; Shen and An 1988; Cole 1989; Chemberlin et al. 2004; Craufurd et al. 2006). Therefore, the peanut producers have tried many practices to lift up the cotyledon node out of the soil surface. At first, they clear the soil around the seedling away to expose the cotyledon node. It is called “Qingke” in Chinese language, meaning “clearing soils around the seedling” (Shen 1958). Then a AnM technique was proposed, i.e., inducing hypocotyls elongation, exposing the elongated hypocotyl to light and dry air, and earthing up soils to welcome the late pegs (Shen and An 1988). The yield increase by AnM technique has been proved to reach up to 30% and some agronomic advantages and the related mechanisms for yield increasing effect by AnM technique have been examined by Shen’s group (Shen and An 1988; Shen 1990) and in my previous experiments. In order to practice the AnM technique easily by machine, Shen et al. (1996) again proposed a simplified AnM technique in combination with film mulching. AnM technique has proved the same effective in addition to the high yield improvement by film mulching technique (Shen et al. 1996). The previous experiment of the present research proved that the yield increase by a black bio-degradable film reached up to 47% and the AnM technique resulted in a yield increase of 16% over the 47% yield increase by film mulching in Matsumoto highland area with a cold spring weather. The added effect of AnM plus film mulching reached 70%. The mechanisms for the yield improvements were clarified by analyses of the pressure-volume curve and the excised leaf transpiration declining curve. The results suggested that both AnM and film mulching techniques induced xerophytophysiological regulations such as leaf turgor improvement by osmotic adjustment and water loss avoidance by strengthened leaf surface structure. However, the osmotic adjustment was confirmed through indirect analysis such as the P-V curve

analysis and it was not clear whether concentration of the related solutes really increased or not. Therefore, in the present experiment, concentrations of some solutes related with osmotic adjustment, such as sugars and proline as well as the protein which would be related with the amino acids, were measured in addition to the P-V curve analysis. In the previous experiment, with the design coped with nature farming philosophy, a bio-degradable black film made of corn starch, which was supposed as no pollution to the environment, was used. However, in the actual production, white, transparent polyethylene plastic films are often used. Because the films are transparent, weeds cannot be suppressed completely as the black film did. The effect of increasing soil temperature might be different between the transparent and black films. Therefore, in addition to confirm the effect of the modified AnM technique, the effect was also compared between the white transparent and black films.

5.3 Materials and Methods

5.3.1 Experiment site

The experiment was conducted on an organic farm located in the suburb of Matsumoto City, Nagano Prefecture, Japan (36°12′N, 137°48′E; 700 m above sea). The climate was characterized by a central highland with annual precipitation of 1016 mm and average high and low temperatures of 17.6 and 6.3ºC.

5.3.2 Soil properties, plant materials and treatments

The soil belonged to Andosol, a kind of volcano ash. The soil properties were the same as described in Chapter 3 (Table 3.2). A biofertilizer fermented using rice bran, soil mill sludge and fish meal as materials was applied 210 g m^{-2} on dry mass basis on April 26, two weeks before sowing. The biofertilizer contained N, P, and K at N, P and K at 40.2, 28.3 and 19.2 g kg^{-1}, respectively. Five days after mulched, seeds of peanut (*Arachis hypogaea* L. cv. Chibahandachi) were sown with the distance 20 cm between holes and 80 cm between rows. A 2×2 factorial experiment was designed with two kinds of mulch as the main plot and AnM technique as the sub-treatment, placed in a 4×4 Latin Square in 4 rainout shelters, each contained 4 plots. The treatments were 1) WA, peanut plants grown with AnM method mulched with white transparent film; 2), WC, white transparent film without AnM; 3) BA, AnM method mulched with bio-degradable black film; and 4) BC, bio-degradable black film without AnM treatment.

5.3.3 Evaluation of disease index

Some plants were infected by the pathogen for the brown leaf spot, *Mycospaerella arachidis*. The disease was indicated by both percentage infection and the infection degree shown by the disease index (DI). The percentage infection of the plants was calculated from 20 plants in each plot. Disease index was estimated as DI = ∑(Number of infected leaves to a certain degree × Degree coefficient)/(total sample leaf number × highest degree coefficient). The degree coefficient was scored from 0 (no symptom. through 1 (12.5% of the leaf area was infected), 2 (25%), 3 (50%) and 4 (75%) to 5 (completely infected). Percentage of the rotting shells, which were contaminated by aflatoxin, was also recorded.

5.3.4 Measurements of leaf photosynthesis, leaf color and pressure-volume curve

Analyses of leaf photosynthesis and pressure-volume referred to Materials and Methods 2.3.3 and 2.3.4, respectively, in the Chapter 2.

The leaf color was measured using a chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Konica Minolta Sensing, Inc., Tokyo, Japan). The readings by the non-destructive, quick and easy measurement indicate the chlorophyll content of the plant leaves.

5.3.5 Analysis of plant growth and leaf water retention

Plant growth and leaf water retention analysis was made as described in Materials and Methods 3.3.6 and 3.3.11, respectively, in Chapter 3.

5.3.6 Measurements of soluble proteins, soluble sugars and proline

The main osmotic solutes in mesophyte plants are sugars, especially sucrose, glucose and fructose, and amino acids, especially proline.

Total soluble proteins (or the activated enzyme solution) were extracted by pH 7.5 50 mM phosphate buffer solution and determined according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford 1976; Murphy et al. 1989). Standards were prepared as a range of 0 to 150 μg protein (BSA). Its absorbance at 595 nm was determined after 2 min and before 1 hour using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The content of soluble proteins was expressed as μg per g of fresh weight.

The measurement of soluble sugars was according to the anthrone-sulfuric acid method (Somani et al. 1987). Carbohydrates, such as soluble sugars, are dehydrated by concentrated sulfuric acid to form furfural, which condenses with anthrone to form a blue-green colored complex solution. Sample of 0.2 g hypocotyl or leaf of peanut seedlings was added with 5 ml distilled water, covered with parafilm and incubated in boiling water for 30 min. The extracted solution was diluted with water to set the volume to 25 ml. The reaction was as follow: 0.5 ml of sugar solution, 1.0 ml distilled water, and 0.5 ml of saturated anthrone solution (ethyl acetic saturated with anthrone) and 5 ml of concentrated sulfuric acid are added. The reaction was under boiling water for 10 min, cooled down to room temperature. The solution shows the maximum absorption at 620 nm. Glucose was used to make the standard regression curve under different content of glucose. The reaction was the same as the above mentioned. The soluble sugar content is expressed as mg per g of fresh weight.

Proline colorometric determination was conducted according to Bates et al. (Bates et al. 1973; Marin et al. 2010) based on the reaction of proline with ninhydrin. Ninhydrin reacts in particular with proline, an imine in which the amine group has cyclized with the side chain to form colored substance. Sample of about 0.3 g hypocotyl or leaf was used for proline extraction in 5 ml 3% sulfosalicylic acid solution for 10 min at 100˚C. A 1:1:1 mixture of extracted proline solution, glacial acetic acid and 2.5% acid-ninhydrin was incubated at 100˚C for 30 min. The reaction terminated in an ice bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The proline content was expressed as μg per g of fresh weight.

5.4 Results

5.4.1 Both the black and white mulching suppressed weed incidence

Peanut plants mulched with bio-degradable black film showed deeper color in green (SPAD value in Table 5.3) throughout the growth period. Peanut plants in AnM technique were also deeper in green than those in non-AnM treatments but the difference was not so clear as between two kinds of mulches. Weeds throve under the white transparent film although the soil surface was mulched closely. However, the weeds grew only on both sides of the peanut plant row a little away from the plants or no weeds grew under or into plant stands (Fig. 5.1). It was the peanut plant stands instead the white film that suppressed the weeds in the place close to plant stands. The weeds could be easily deleted by hand weeding or machine weeding. However, if the pegs had penetrated into the soil where the weeds throve, weeding should be avoided in order not to damage the pegs. In the present experiment, the weeds were easily deleted by hands. Black film mulching completely suppressed the weeds (Fig. 5.1). The weeds were restricted under the film supposed with little effect on peanut plant growth. The greener color of leaves of peanut plants in black film plots might be attributed to improved nutrient mineralization because of more heat was absorbed by the black film than by the white transparent one. Weeds might competitively absorbed N in the white film mulch and make the plants appearing in less green color.

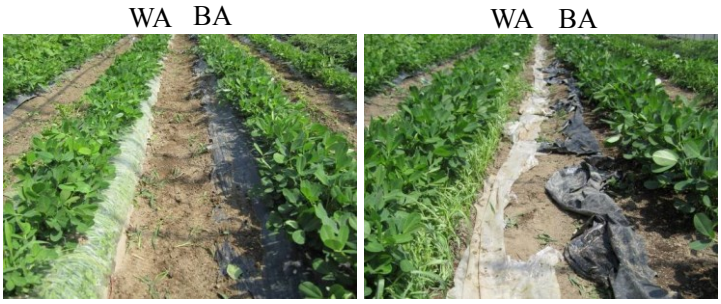

Fig. 5.1 Weeds were suppressed by black film mulching (right in each photo) but not by the white transparent film (left in each photo). WA, AnM method mulched with white transparent film; BA, AnM method mulched with bio-degradable black film.

5.4.2 AnM mulched with two types of film increased biomass production and yield components

Dry mass per plant was higher in AnM than in non-AnM treatment for both kinds of film mulching and was also higher in black film treatment than in white film treatment for both AnM and non AnM treatments (Table 5.1). Dry mass of total pods and marketable pods showed similar trends to that of total dry mass per plant with exception that the difference in marketable pod dry mass was not significant between white and black film treatments. AnM increased shell number with no difference between white and black film mulching. Shell size was similar among treatments. Shell yield per unit area of farmland was higher in AnM than in non-AnM treatment with a significant interaction between AnM and mulch type. The difference in shell yield was not significant between white film and black film plot when AnM technique was adopted. The harvest index showed a trend reversely proportional to shell yield, i.e., a higher shell yield with a lower harvest index.

5.4.3 AnM combined with mulching showed much effective in preventing disease and rot shells than mulching only

Peanut leaves were infected by brown leaf spot (*Mycospaerella arachidis*) at the later growth stages and the disease incidence was shown by percentage infection of plants and disease index (DI), which showed the degree of the infection. Percentage of infected plants and DI were both lowered by AnM with small or no difference between white and black film treatments (Table 5.1). Rot pods from the early flowering might be contaminated by aflatoxin and further pollute the shell product and lower the product quality. Rot pods were found in treatments of both kinds of film mulching without AnM but almost not found in AnM plots. AnM technique prevented formation of early pods by controlling early flowering and early penetration of the pegs. This result was consistent with the previous experiments of the present research.

Table 5.1 Dry mass and yield components of peanut plants.

Treatment	--------Dry mass (g pl^{-1})--------			Shell no. per plant		Shell size (g)		Yield	H. index	Disease (%)		Rot pod
	Plant	Pods	Mark. pods	Total	Mark.	Total	Mark.	(g m^{-2})	(%)	Plant	Index	(%)
WA	130.8	61.9	57.1	33.2	26.6	1.9	2.1	393.4	43.7	13.4	3.4	0.3
WC	116.7	55.0	52.8	28.7	22.7	1.9	2.3	368.0	45.2	26.7	7.2	1.6
BA	143.4	65.2	58.7	35.0	27.2	1.9	2.2	419.3	40.9	17.5	4.6	0.2
BC	126.8	59.8	54.1	28.9	24.0	2.1	2.3	386.9	42.7	22.6	5.8	1.4
Mulch	*	*	ns	ns	ns	ns	ns	*	*	*	*	ns
AnM	*	*	*	**	*	ns	ns	**	*	**	*	**
M×A	ns	ns	ns	*	ns	ns	ns	*	ns	*	ns	ns

Mark., marketable; H. index, harvest index (%) calculated as the percentage ratio of marketable pod dry mass to total plant dry mass. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12). WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment.

5.4.4 AnM mulched with black and white film increased plant growth dynamics

The dynamic curves of plant growth showed clear differences when AnM was applied, while similar between white (WC) and black (BC) mulches (Fig. 5.2). The parameter g_M showed the maximum increment of dry mass per plant during the growth period and was increased by AnM treatment with a significant synergistic interaction between AnM and mulch type (Table 5.2). The value of g_M was higher in black mulch plot if the AnM was adopted but showed no difference between mulch types if AnM was not adopted. It was suggested that black film was better in terms of plant growth improvement than white transpiration film when used in the modified AnM technique. The parameter g_O was dry mass per plant at the beginning of the growth period and theoretically should be different between treatments. The coefficient α was related with the growth rate. In the present experiment, the value of α showed no difference among the four treatments. AnM shortened the day needed for the plant growth to reach half of the maximum dry mass (τ) when combined with the black mulch but showed no effect when mulched with white film. In overall, the main difference of plant growth dynamics was in the maximum dry mass increment (g_M).

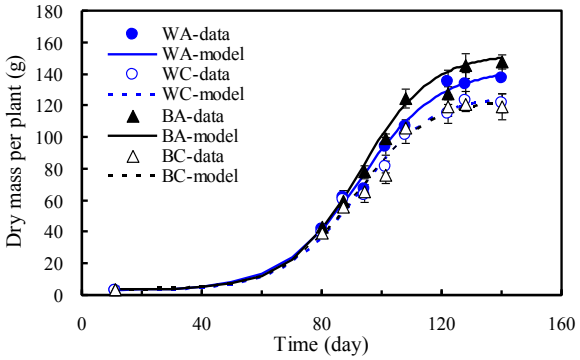

Fig. 5.2 Growth dynamic curves for peanut plants. data, the average of original data (n=12); model, the models analyzed by $g = g_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-g_B(1-\beta t)$ according to the original data. WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. Error bars represent SE values for twelve independent replicates.

Table 5.2 Parameters from the analysis of growth dynamic curve.

Treatment	g_M (g per plant)	g_B (g per plant)	β	α	τ (day)
WA	140.6	3.0	0.00308	0.074	95.5
WC	124.4	3.3	0.00309	0.078	96.5
BA	151.3	3.6	0.00346	0.076	96.5
BC	122.2	3.9	0.00336	0.076	99.5
Mulch	ns	*	*	ns	*
AnM	**	*	ns	ns	*
M×A	*	ns	ns	ns	*

g_M, the maximum increment of plant dry mass; g_{Be4}, the original biomass of the plant at the beginning; β and α, constants; τ, the time point when dry mass reached half of g_M. WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. *, ** and ns mean significance at $P\leq0.05$, at $P\leq0.01$ and no significance according to LSD analysis.

5.4.5 AnM method mulched with black and white film improved photosynthetic activities

Throughout the whole growth period, the net leaf photosynthetic rate (P_N) was higher in AnM than in non-AnM treatment and also slightly higher in black film than in white film mulch (Fig. 5.3). Both the maximum photosynthetic capacity (P_C) and maximum quantum use efficiency (Y_Q) was increased in BA and WA treatments where AnM was adopted but showed little difference between BA and WA (Table 5.3). Without AnM, black and white mulching had similar effect on P_C and Y_Q. The coefficient K was quantitatively equal to the reciprocal of the PPF at $P_N = 0.63\ P_C$, showed the curvature of the curve, and was also related to the light saturation status of the photosynthesis. Values of Y_Q and K were proportional to photochemical activity in the whole photosynthetic process and thus also related the chlorophyll in a limited range. In the present experiment, both Y_Q and K in black film treatment did not show higher values than those in white film treatment despite of higher chlorophyll concentration shown by SPAD values of the leaf color in the black film treatment since the leaf chlorophyll concentration was at higher levels in all the treatments. PPF_C, the light intensity when photosynthesis just compensated the consumption of respiration, was lowered by AnM and also lower in black film than in white film treatment. The value of PPF_C was reversely proportional to photochemical activity and thus consistent with the value of Y_Q in the present experiment, suggesting an increasing effect on photochemical activity by AnM treatment.

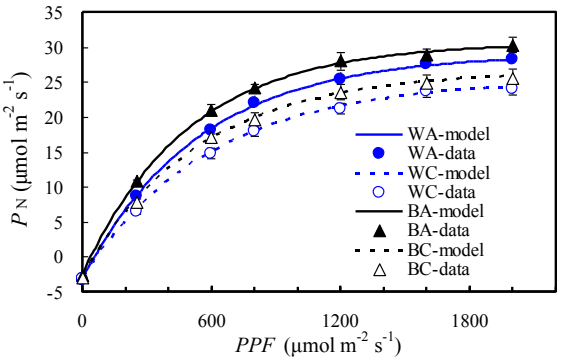

Fig. 5.3 Photosynthetic light response curves for peanut plants. WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. data, the average of original data (n=12); model, the models analyzed by $P_N = P_C (1\text{-}e^{-KI}) - R_D$ according to the original data.

Table 5.3 Parameters from the analysis of photosynthetic light response curve for peanut plants grown.

Treatment	P_C	R_D	K	Y_Q	PPF_C	Leaf color
	--(μmol m^{-2} s^{-1})--		(m^2 s μmol^{-1})	(mol mol^{-1})	(μmol m^{-2} s^{-1})	SPAD
WA	32.3	3.3	0.00202	0.0653	58.4	49.4
WC	28.5	3.2	0.00186	0.0528	70.3	47.2
BA	33.2	2.6	0.00183	0.0612	40.5	53.2
BC	29.5	2.9	0.00169	0.0501	55.8	51.1
Mulch	*	*	*	*	**	**
AnM	*	ns	**	**	**	*
M×A	ns	ns	*	*	**	*

P_C, photosynthetic capacity; R_D, dark respiration rate; Y_Q, the maximum quantum yield; K, the half time constant with the maximum quantum (Y_Q) defined as $Y_Q = KP_C$; PPF_C, compensation point defined as PPF at P_N=0.

WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis.

5.4.6 AnM with black mulching improved cell water condition by osmotic adjustment

The leaf water potential at full turgor or at the saturated leaf water status (Ψ_{FT}) showed no difference among treatments. When AnM was adopted in black and white mulching, the osmotic potential at full turgor (π_{FT}) was lowered compared with mulching only without AnM (Table 5.4). π_{FT} was more minus in black film treatment than in white film treatment. The parameter π_{s+a} was osmotic potential in the symplastic solution theoretically diluted by apoplastic water, showed a similar trend to π_{FT}. The turgor potential was the difference between water potential and osmotic potential. Therefore, the turgor potential at water-saturated conditions (P_{FT}) was higher in AnM with mulching (BA and WA) treatments than in treatments mulched only (BC and WC). It seemed that black film mulching had a little higher effect on the increase of turgor potential (P_{FT}) than white film. Difference in leaf water potential at midday (Ψ_{MD}) among treatments was very small but the osmotic potential at midday (π_{MD}) was lowered in AnM with mulching treatments than in treatments without AnM. Consequently, P_{MD} was increased by AnM and black and white mulching showed same effective. The improved leaf turgor potential was accountable for the improvements in photosynthetic activities and plant growth by AnM and film mulching treatments since leaf turgor potential was the driving force for plant to grow and for stomata to open in photosynthetic process. Improved leaf turgor potential was resulted from osmotic adjustment shown as the further lowered osmotic potential on the basis of similar leaf water potentials. The osmotic concentration (C_{FT}) and the difference of osmotic potential or the osmotic concentration (ΔC_{FT}) over the control value showed the active increase in osmotic molecules caused by AnM and a little active increase by black mulching.

The osmotic potential at incipient plasmolysis (π_{IP}), when the cell wall would separate from the symplasm because of the symplasm shrink, was lower in AnM than in non-AnM treatments and also slightly lowered by black film mulching, suggesting a higher tolerance to cell dehydration in AnM treated peanut plants. The leaf or cell relative water content at incipient plasmolysis (ζ_{IP}) showed a similar trend to π_{IP}. Results of π_{IP} and ζ_{IP} showed that AnM and film mulching, especially the black one, improved water stress tolerance in peanut plants.

The symplastic water fraction (ζ_{sym}) was larger and the apoplastic water fraction (ζ_{apo}) was smaller in AnM than non-AnM plots with no difference between white and black film. It was suggested that AnM technique improved cell water conditions in the symplasm where biochemical functions were performed. Water that is more compartmented in symplasm favorites biochemical functions and cell turgor maintenance, especially in case of water deficit (Patakas and Noitsakis 1997). For most parameters, there existed a synergistic interaction between AnM treatment and mulch film type, always with a better effect of AnM in black film mulching.

Table 5.4 Parameters from P-V curve analysis for peanut plants.

Treat.	Ψ_{FT}	Ψ_{MD}	π_{FT}	π_{MD}	P_{FT}	P_{MD}	π_{s+a}	π_{IP}	α	β	ζ_{IP}	ζ_{sym}	ζ_{apo}	C_{FT}	ΔC_{FT}
	----------(MPa)----------								--($\times 10^{-3}$)--					-(osmol m^{-3})-	
WA	-0.23	-0.86	-1.51	-1.79	1.28	0.93	-1.27	1.73	55.62	0.906	0.906	0.825	0.175	709.9	101.9
WC	-0.19	-0.85	-1.25	-1.62	1.07	0.77	-1.01	1.47	49.49	0.921	0.852	0.804	0.196	608.0	0.0
BA	-0.24	-0.83	-1.57	-1.77	1.33	0.94	-1.33	1.79	57.78	0.942	0.942	0.818	0.182	737.4	129.5
BC	-0.20	-0.82	-1.38	-1.53	1.18	0.71	-1.11	1.63	54.54	1.015	0.939	0.784	0.216	670.0	62.0
Mulch	ns	*	*	*	*	*	*	*	*	*	*	ns	ns	*	**
AnM	ns	ns	*	**	**	**	**	**	*	*	*	*	*	**	**
M×A	ns	ns	*	*	*	*	*	*	ns	*	*	*	*	*	**

The P-V curve was modeled as follow,

$-\Psi^{-1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]$.

Ψ_{FT}, leaf water potential at full turgor or in saturated leaf water condition; Ψ_{MD}, leaf water potential at midday; π_{FT}, osmotic potential at full turgor; π_{MD}, osmotic potential at midday; π_{s+a}, the osmotic potential in the symplastic solution theoretically diluted by apoplastic water; π_{IP}, osmotic potential at incipient plasmolysis; P_{FT}, leaf turgor potential at full turgor; P_{FT}, leaf turgor potential at midday; P_{MD}, leaf turgor potential at midday; ζ_{apo}, apoplastic water fraction shown as $\zeta_{apo} = \zeta_{FT}-\zeta_{sym} = (1-\zeta_{sym})$, where, ζ_{sym} is the symplastic water fraction; ζ_{IP}, relative leaf water potential at incipient plasmolysis; β and α, constants; C_{FT}, osmotic concentration at full turgor; ΔC_{FT}, increment of osmotic concentration caused by active osmotic adjustment compared with the control.

WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis.

5.4.7 Leaf water retention ability was increased by AnM and mulching

The value of the coefficient α, which was quantitatively proportional to the stomatal transpiration level, was larger in the excised leaves of peanut plants in AnM treatments (BA and WA) than in treatments without AnM (BC and WC) (Table 5.5). The leaf relative water content (ζ_{SC}) when stomata closed was slightly lower by AnM. This implied that peanut plants in AnM were suggested more physiologically tolerant to leaf water deficit because they could open stomata to maintain photosynthesis under leaf water deficit conditions compared with those in control plots. The time when stomata got closed (t_{SC}) showed no significant difference between AnM and non-AnM treatments but black film mulching prolonged the time for stomata close. The values of β, which was proportional to the level of cuticular transpiration, were lower in AnM than in non-AnM treatments with no difference between white and black film mulching. This suggested that leaves of peanut plants in AnM might have prevented water loss from the cuticular membrane by developing thicker cuticles and/or depositing more wax on the leaf surface. The constant τ_D, which represented the time when the leaf is air dried to remain 10% of relative leaf water content, was prolonged by AnM with no significant difference between white and black film. In the present experiment and the previous experiments, peanut plants were healthier and stronger morphologically in AnM than in non-AnM control. The improved morphology and leaf surface structure might also have prevented abovementioned disease infection.

Table 5.5 Variables from the analysis of excised leaf transpiration declining curves.

Treatment	ζsc	t_{SC} (min)	α	β	τ_D (min)
WA	0.793	2.99	0.889	0.0080	108.2
WC	0.847	2.80	0.648	0.0112	91.4
BA	0.714	3.66	0.842	0.0078	112.2
BC	0.790	3.75	0.562	0.0109	87.7
Mulch	*	*	*	ns	ns
AnM	*	ns	**	**	**
M×A	*	ns	*	ns	ns

Leaf water retention ability was evaluated by $\zeta=[\zeta_{sat}-\zeta_{SC}(1-\beta t)]\ e^{-\alpha t}+\zeta_{SC}(1-\beta t)$.

ζ_{SC}, leaf relative water content at the time when the stomata closed; β, a constant related to cuticular transpiration; α, a constant related to the stomatal transpiration; t_{SC}, time when stomata started to close; τ_D, time used to dry the excised leaf to a relative water content of 0.10. WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. *, ** and ns mean significance at $P\leq0.05$, at $P\leq0.01$ and no significance according to LSD analysis.

5.4.8 AnM and the mulching actively increased osmolytes

Sugars such as sucrose, glucose and fructose and amino acids such as proline are the main osmotic solutes in the cell symplasm (Sánchez et al. 1998). Especially, proline concentration is closely related with environmental stimulation (Handa et al. 1983). In the present experiment, concentration of sugars in leaves of peanut plants was increased in AnM treatments 3 days after the hypocotyls were exposed to light and dry air with no significant difference between white and black film mulching (Table 5.6). Even 30 days passed after hypocotyl exposure treatment, these differences in leaf sugar concentration were still clear and significant.

Hypocotyl exposure in AnM treatments (BA and WA) significantly increased proline concentration at day 3. Thirty days after the hypocotyl exposure, proline concentration decreased but still higher in BA and WA treatments. In addition, proline was also induced high in black film mulching.

Concentration of proteins was lowered soon by hypocotyl exposure in AnM, but this difference reversed even though it was small 30 days after hypocotyls were exposed. The proteins might breakdown into osmotic amino acids in response to the stimuli and were compensated by the regulation mechanisms one month later. The results of direct measurement of the osmotic solutes confirmed the results of P-V curve analysis. In overall, it was suggested that active increases in osmolytes decreased the leaf osmotic potential on the basis of a similar leaf water potential and consequently maintained a high leaf turgor potential, which in turn improved leaf photosynthesis and plant growth.

Table 5.6 Concentrations (mg kg^{-1}) of osmotic solutes such as sugars and proline as well as soluble proteins.

Treatment	-----Sugars-----		------Proline------		-----Proteins-----	
	3d	30d	3d	30d	3d	30d
WA	19.2	21.7	87.6	32.7	14.8	16.7
WC	15.4	17.8	39.2	14.6	19.4	15.2
BA	18.3	22.6	93.6	39.4	16.9	21.6
BC	15.1	18.7	45.8	28.6	21.4	19.1
Mulch	*	**	**	**	*	*
AnM	*	*	**	**	*	*
M×A	*	*	*	*	ns	*

Measurements were made at 3 days and 30 days after the hypocotyls were exposed to light and dry air. WA, AnM method mulched with white transparent film; WC, white transparent film without AnM; BA, AnM method mulched with bio-degradable black film; BC, bio-degradable black film without AnM treatment. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=3).

5.5 Discussion

Film mulching technique has been vast adopted in peanut production in China and other Asian countries (Wang and Han 1986). The expected and already confirmed effects of film mulching are 1) increasing soil temperature, 2) maintaining soil moisture, and 3) controlling weeds (Ramakrishna et al. 2006). The shell yield increase is in a range of 750 to 1500 kg per ha or in percentage range of 16-26% in the scale of large area production. In my previous experiment, it was found that the yield improvement by mulching a biodegradable black film the same as used in the present experiment reached 44% and the yield increasing effect by film mulching was higher than that by any other agronomic technique. This might be because that fertilizer was all organic and the spring was cold in Matsumoto highland area. The film mulching warmed up the soil and increased mineralization of the organic fertilizer. In addition to the base of film mulching technique, Shen et al. propose a modified AnM technique, whereby the normal AnM technique was combined with the film mulching technique (Shen et al. 1996). In my previous experiment, it was found that the shell yield was significantly increased by both AnM and mulching treatments with significant synergistic interaction. Even on the base of such a high yield increment, AnM treatment still increased shell yield by 19% in addition. The combined yield increasing effect of AnM and film mulching reached as high as 71%. At the beginning, both AnM and film mulching techniques were adopted as agronomic practices with the expected effects for film mulching as increasing soil temperature, maintaining soil moisture and suppressing weeds, and for AnM technique as sending the cotyledon node out of the soil surface by the elongated hypocotyl. However, at the beginning, the effects were not expected from environmental stimulation, signal transduction and the xerophytophysiological regulations. A theory of applying xerophytophysiology and signal transduction in plant production was proposed (Xu 2007). According this theory, the expected consequences such as high sugar content of fruit, healthy crop and improved crop yield can be induced by imposing an artificial stimulus to the un-stressed plants, which perceive the environmental stimulus, send the signal to the internal gene systems, activate some related genes and lead to the expected regulations (Mulligan 1997; Smith and Gallon 2001). The theory of xerophytophysiology has been applied to tomato crops by restricted irrigation (Xu et al. 2010), low air humidity (Xu et al. 2007) and canopy blue light irradiation (Xu et al. 2011b), to sorghum by exposing the mesocotyl (Xu et al. 2009d), to garlic by exposing the cloves (Qin et al. 2008a), and to potato by partial rootzone drying (Xu et al. 2011a). Actually, both AnM and film mulching techniques belong to applications of xerophytophysiology in plant production. AnM treatment induced osmotic adjustment by the stimulus of exposing the hypocotyls to light and dry air, where the hypocotyls usually grow in soil instead exposed. Mulching creates conditions of low humidity, mild dry soil and high soil temperature and low air humidity above the mulch (Ramakrishna et al. 2006). For example, a small and low

film tunnel with the soil mulched on the soil surface can create a desert air conditions for water crop in side the tunnel but the water was sufficiently from the roots. The dry conditions in the tunnel did not cause a real drought.

In my previous experiment, it was confirmed that mulching with biodegradable black film also induced osmotic adjustment that accounted for improvements in photosynthetic activities, plant growth and shell yield of the peanut crop. However, in practice of peanut production, most used are white transparent films instead the black ones. Of course films used in combination with AnM technique are also the white transparent ones. In the present experiment, effect of AnM was compared between using white and black films and it was found that mulching by white and black film both induced xerophytophysiological regulations whereby photosynthesis, plant growth and shell yield were improved. The black film mulching completely suppressed weeds but the transparent film could not completely suppress weeds. The weeds throve along both sides of the peanut crop row. The yield increasing effect was also slight lower in white film mulching than in black film.

In conclusion, black film was better than the white transparent one in peanut production, especially when combined to the AnM technique. However, in case the manufacture of the biodegradable film cannot sufficiently meet the demand in agricultural production, the white transparent one can also be a good option. The AnM technique was equally effective in combination with both the transparent film and the black film.

Chapter 6

Seedling Transplanting as the Alternative of AnM Technique in Peanut Production

6.1 Abstract

Improvements in plant growth and shell yield of peanut crops have been proven by the AnM technique including hardening the seedlings by exposing hypocotyls as stimulation. However, practices of AnM technique in fields, such as inducing hypocotyl elongation and exposing the elongated hypocotyls, are usual labor-cost and not easy for mechanization. In the present experiment, transplanting of seedlings with elongated hypocotyls was tried as an alternative of AnM technique and mechanisms for xerophytophysiological regulations such as osmotic adjustment and morphological strengthening were clarified. Four treatments were designed in a 2×2 factorial as 1) T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; 2) T-Control, seedlings with un-elongated hypocotyls; 3) S-AnM, direct seeding with hypocotyls exposed by removing the cover soils; and 4) S-Control, direct seeding without hypocotyl exposing treatment. Both seedling transplanting and AnM treatments improved plant growth, photosynthetic activities and shell yield of the peanut crop. The shell yield was increased 11.7% by seedling transplanting, 20.3% by field AnM practice, and up to 33.7% by the additive effect of seedling transplanting and AnM treatment. Seedlings were hardened and plants were strengthened by exposing hypocotyls. Xerophytophysiological regulations such as osmotic adjustment were induced by AnM treatment and also by the transplanting practice, which were confirmed by the analysis of pressure-volume curve. Leaf osmotic potential was lowered due to the solutes concentration increase and leaf turgor potential was improved by seedling transplanting and AnM treatments. Symplastic water fraction was increased by the both AnM and seedling transplanting. Analysis of excised leaf transpiration curve confirmed that both AnM and seedling transplanting reduced cuticular transpiration water loss by strengthening of leaf surface structure. In conclusion, transplanting of seedlings with elongated hypocotyls could be the alternative of hypocotyl exposing in the AnM technique and show similar positive effects in peanut crop production.

Keywords: AnM technique, seedling transplanting, osmotic adjustment, peanut (*Arachis hypogaea* L.), xerophytophysiology.

6.2 Introduction

A technique called AnM method has been adopted in peanut production in China (Shen and An 1988). Later, a modified AnM technique in combination with film mulching was proposed by Shen et al. (1996) to simplify the AnM practices. At the beginning, the purpose of AnM technique by inducing hypocotyl and removing away the soil around at the "n" stage was to lift up the cotyledon node. Otherwise the cotyledon node would remain in soil, the first two branches from the cotyledon node would produce pods, and the early pods would compete for nutrition with the whole young plant and rot before harvest. A hypothesis was proposed in my previous experiments that exposing the hypocotyl in AnM method was a xerophytophysiological stimulation, which could induce signal transduction and xerophytophysiological regulations and make the peanut plant healthier than usual, in addition to the agronomical advantages. The physiological mechanisms and consequences for improvements in plant growth and yield by AnM techniques include leaf turgor maintenance by osmotic adjustment, increased symplastic water fraction, strengthened leaf surface structure and enhanced resistances to disease, pest insect and adverse environmental conditions. However, both the normal and modified AnM techniques are labor-cost and unfeasible for mechanization. Therefore, in the present experiment, transplanting of seedlings with elongated hypocotyls was tried as an alternative of AnM techniques and comparisons were made between seedlings with and without extra elongated hypocotyls and between seedling transplanting and direct seeding. The agronomical performance and the physiological fundamentals were examined.

6.3 Materials and Methods

6.3.1 Culture of seedlings

Seeds of peanut (*Arachis hypogaea* L. cv. Chibahandachi) were sown in seedling packs. The packs were placed in a growth incubator, where the temperature and air humidity were controlled at 25˚C and 60% under 14/10 hours of day/night. When the seeds immerged and the hypocotyl elongated to about 5 cm long, hypocotyls were exposed by removing the soil and seedling packs were moved to a lighting growth chamber where the temperature and air humidity were the same as in the dark incubator and the light intensity was 180 μmol m^{-2} s^{-1} over the canopy of the seedlings. After one week with hypocotyl exposure, seedlings were transplanted. As the control, seedling emerged naturally without hypocotyl exposed before seedling transplanting.

6.3.2 AnM treatments in field

Shallow ridges 5 cm high were produced in the experimental field under rainout shelters and a kind of bio-degradable black film was mulched on the ridges. The seeds were sown in the experimental field the same day as the seeding for seedling culture in growth chambers. The seeds were sown 3 cm deep under the film and a 5 cm high soil mound was made over each seed to induce the extra elongation of the hypocotyl. In the control, there were no soil mounds over the seeds. The following four treatments were designed in a 2×2 factorial: 1) T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; 2) T-Control, transplanting of seedlings with un-elongated hypocotyls; 3) S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; and 4) S-Control, direct seeding without hypocotyl exposing treatment (Fig. 6.1).

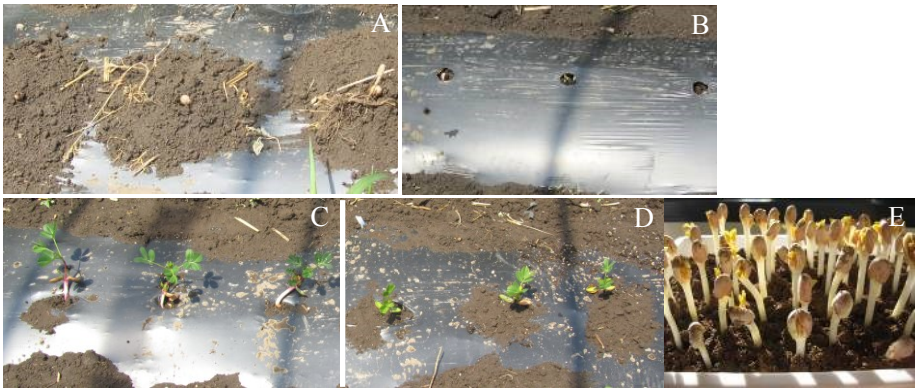

Fig. 6.1 Comparisons between modified and transplanting-alternative AnM techniques in peanut cultivation. A, S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; B, S-Control, direct seeding without hypocotyl exposing treatment; C, T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; D, T-Control, transplanting of seedlings with un-elongated hypocotyls; E, seedlings with hypocotyl exposed in growth chamber.

6.3.3 Soil condition and fertilization

The experiment was conducted in an organic field without chemical fertilizer and pesticide applications. The soil was a kind of volcano ash (Andosol) and was characterized by pH, 6.0; EC, 0.06 mS cm^{-1}; total C and total N 38.3 and 3.1 g kg^{-1}, respectively, NH_4-N, NO_3-N, P, K, Ca and Mg, 6.4, 5.2, 173.2, 339.5, 2643.6 and 441.4 mg kg^{-1}, respectively; and CEC (cation exchange capacity), 18.6 cmol kg^{-1}. A kind of organic fertilizer produced with rice bran, oil mill sludge and fish meal as materials (N, P, and K were 42.4, 30.7 and 21.6 g kg^{-1}, respectively) was applied 200 g m^{-2} before film mulching. Irrigation was made with spraying pike over the crop under the rainout shelter.

6.3.4 Evaluation of biomass production, shell yield and disease index

The final samples were taken and oven dried with shoot and pods separated and the biomass production was evaluated. The diameter and length of the hypocotyl were recorded. Air dried shell samples were used to estimate shell yield and the percentage of the rot pods was calculated. In some of the plants, brown leaf spot was caused by *Mycospaerella arachidis*. Disease index was evaluated as the same as Materials and Methods 3.3.8 in Chapter 3.

6.3.5 Analysis of plant growth dynamics

Plant samples were taken at intervals of 7-10 days and oven dried. The dry mass of shoot and pods was recorded separately. The dynamic changes of the dry mass of shoot and pods referred to Materials and Methods 3.3.6 in Chapter 3.

6.3.6 Measurements of leaf photosynthesis and leaf color

The measurements of the leaf photosynthesis and leaf color were the same to Materials and Methods 2.3.3 in Chapter 2 and 3.3.7 in Chapter 3.

6.3.7 Estimation of osmotic adjustment by pressure-volume curve analysis

Osmotic adjustment and cell water compartment were estimated by analyzing the Pressure-Volume (P-V) Curve as described in Materials and Methods 2.3.4 in Chapter 2.

6.3.8 Analysis of leaf water retention ability from excised leaf transpiration declining curve

Water retention ability was evaluated by analyzing the excised leaf transpiration declining curve as described in Materials and Methods 3.3.11 in Chapter 3.

6.4 Results

6.4.1 AnM with seedling transplanting showed more effect on biomass productivity and shell yield

Transplanting of peanut seedlings with extra-elongated hypocotyls (T-AnM) increased plant biomass and shell yield by 19% compared with the plants using seedlings with un-elongated hypocotyl (T-Control), and by 33.7% and 11.1% respectively, compared with the plants from direct seeding (S-control) and direct seeding of modified AnM (S-AnM) practiced (Table 6.1). Compared with directly seeded plants without AnM treatment (S-control), transplanting seedlings even with un-elongated hypocotyl also increased pod biomass and shell yield although it did not increase the whole plant biomass. If it was compared with the plants from direct seeding without AnM treatment (S-control), transplanting of peanut seedlings with extra elongated hypocotyls increased plant biomass and shell yield by 23.7%, 33.3% and 33.6% respectively. AnM treatment increased pod number but decreased the pod size, which suggested that the yield increase was attributed to the increased pod number instead to the pod size, while yield increase by seedling transplanting was attributed to the increased pod size instead to the pod number per plant. Increases in harvest index by both AnM treatment and seedling transplanting reached significant level although the increments were small. Both diameter and length of the hypocotyl part in the plant at harvest time were enlarged by AnM treatments, although seedlings were artificially transplanted one week after hypocotyls exposure in ST-AnM treatment.

6.4.2 Seedling transplanting with AnM technique decreased disease and rot shell

Peanut leaves of some of the plants were infected by brown leaf spot caused by *Mycospaerella arachidis* at the later growth stages. Disease index was used to show degree of the infection because only percentage of infected plants could not describe the severity of the disease. Percentage of infected plants and disease index were both lowered by seedling transplanting and AnM treatment (Table 6.1). Rot pods were fewer in seedling transplanted and AnM treated plants than their control.

Table 6.1 Yield and disease index in peanut plants.

Treatment	Dry mass (g pl^{-1})		Yield	Pod no.	Pod size	H.I.	Hypocotyl (cm)		Disease (%)		Rot pod
	Plant	Pods	(g m^{-2})	(pl^{-1})	(g pod^{-1})	(%)	Diameter	Length	Plant	Index	(%)
T-AnM	101.8	40.4	444.8	22.2	1.82	0.40	0.66	4.27	8.7	2.6	0.1
T-Control	87.7	34.0	373.6	17.0	1.99	0.39	0.61	3.20	14.6	7.3	0.8
S-AnM	94.3	36.4	400.3	21.2	1.72	0.39	0.65	5.00	18.4	9.4	1.2
S-Control	82.3	30.3	332.8	16.0	1.89	0.37	0.60	3.57	24.2	11.8	1.7
Transplant	**	**	**	ns	*	*	ns	*	**	**	**
AnM	**	**	**	**	*	*	*	**	**	**	**
Trans.×AnM	*	*	*	ns	*	ns	*	*	*	*	**

T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. H.I., harvest index. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=12).

6.4.3 Seedling transplanting fastened the growth of plant and pods

The plants were sampled from the middle growth stage when pod setting started. Before that time, there was no large difference in biomass among treatments. The maximum increment of biomass (G_M) during the examined period for both the whole plant and pods, was higher in transplanted peanut crops than in directly seeded crops and also higher in crops with AnM treatment than in their controls without AnM treatment (Table 6.2). This was consistent with the results of the final biomass production and shell yield. The original biomass (G_B) of the plant at the beginning of the examined period for both the whole plant and pods was higher in seedling transplanting treatments (T-AnM and T-Control) than in treatments with direct seeding (S-AnM and S-Control), because seedlings transplanted to the field grew fast. The time constant α was related with growth rate. A large value of α in seedling transplanting treatments (T-AnM and T-Control) means a fast growth of plant and pods. The parameter τ was the time point at the curve center when the biomass increment reached half amount of G_M. The clearest was the differences in the value of τ, which was smaller or the half time was shorter in transplanted peanut crops than in directly seeded crops and also in crops with AnM treatment than in their controls without AnM treatment. The details for the plant growth during the whole period can be found in the growth dynamic curve together with parameters analyzed from the curve. The analysis showed that the plant growth rate was higher in seedling transplanted crops and in AnM treated crops than in their controls.

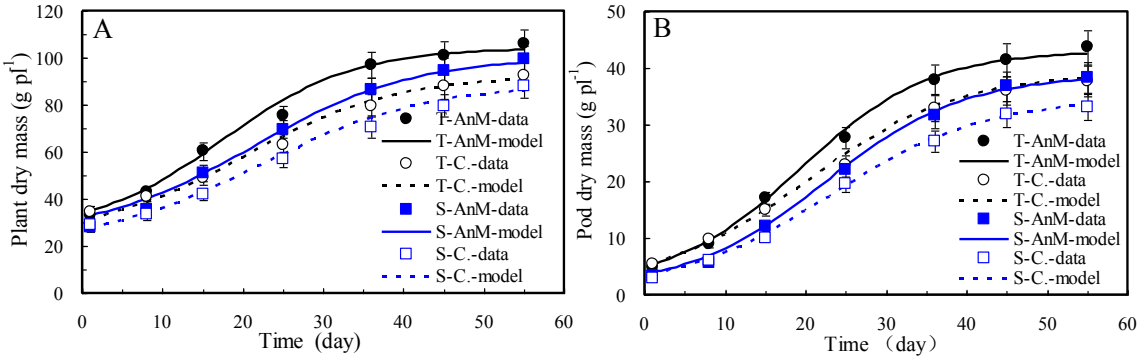

Fig. 6.2 Dynamics model curve of whole plant growth (A) and pod growth (B) for peanut crops. T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. data, the average of original data (n=12); model in A and B, the models analyzed by $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$. Error bars represent SE values for twelve independent replicates.

Table 6.2 Parameters from the growth dynamics for peanut plants.

Treatment	G_M	G_B	α	β	τ
	------(g pl^{-1})------				(day)
	----------For growth of the whole plant----------				
T-AnM	79.2	25.5	0.122	0.00034	18.6
T-Control	71.2	23.5	0.108	0.00022	20.3
S-AnM	72.2	23.5	0.101	0.00019	19.4
S-Control	66.6	21.5	0.098	0.00014	21.5
Transplant	*	*	*	**	*
AnM	**	*	*	**	**
Transplant×AnM	*	ns	**	**	ns
	---------- For growth of the pods----------				
T-AnM	41.6	1.5	0.124	0.00043	19.5
T-Control	37.6	1.5	0.108	0.00036	20.5
S-AnM	37.8	1.0	0.117	0.00033	21.9
S-Control	30.6	1.0	0.107	0.00019	23.8
Transplant	**	**	ns	**	**
AnM	**	ns	**	**	**
Transplant×AnM	*	ns	*	*	*

G_M, the maximum increment of dry mass; G_B, the base dry mass; α and β, constants; τ, the time when the dry mass increment reached half of G_M. T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. *, ** and ns mean significance at $P\leq0.05$, at $P\leq0.01$ and no significance according to LSD analysis (n=12).

6.4.4 Seedling transplanting and AnM practices increased photosynthetic activities and leaf color

The net photosynthetic rate (P_N) in AnM treatments (T-AnM and S-AnM) was higher in all the measured photosynthetic photon flux (*PPF*) than that in treatments without AnM (T-Control and S-Control) (Fig. 6.3). Moreover, seedling transplanting increased P_N. Both seedling transplanting and AnM method increased the capacity of photosynthesis (P_C) (Table 6.3). The difference was not larger in dark respiration rate (R_D) among treatments although it reached significant levels. *K* was the half time constant and related with the curve saturation status and the initial slope of the curve. The seedling transplanting had the same effect on increased the maximum quantum use efficiency or quantum yield (Y_Q) as the AnM method, indicating the high leaf photosynthesis. The results suggested that both seedling transplanting and AnM practices improved the photosynthetic activities in peanut crops.

In the present experiment, the leaf color, which was supposed to represents chlorophyll concentration, was measured nondestructively by a SPAD chlorophyll meter. The data (Table 6.3) showed that both seedling transplanting and AnM practices increased leaf color, which might be, at least partly, contributed to the improvement in photosynthetic activities.

Table 6.3 Parameters of photosynthetic light response curve for peanut plants.

Treatment	P_C	R_D	K	Y_Q	PPF_C	Leaf color
	-(μmol m^{-2} s^{-1})-		(m^2 s μmol^{-1})	(mol mol^{-1})	(μmol m^{-2} s^{-1})	SPAD
T-AnM	33.4	4.1	0.00263	0.0878	49.8	55.8
T-Control	30.8	3.9	0.00252	0.0776	53.7	52.6
S-AnM	31.9	3.7	0.00248	0.0791	49.7	53.4
S-Control	29.2	3.5	0.00234	0.0683	54.6	51.9
Transplant	*	**	*	**	ns	ns
AnM	*	*	*	**	**	*
T×A	ns	ns	ns	ns	ns	*

P_C, photosynthetic capacity; R_D, dark respiration rate; K, the half time constant with the maximum quantum (Y_Q) defined as $Y_Q = KP_C$; PPF_C, compensation point defined as PPF at P_N=0. T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=3).

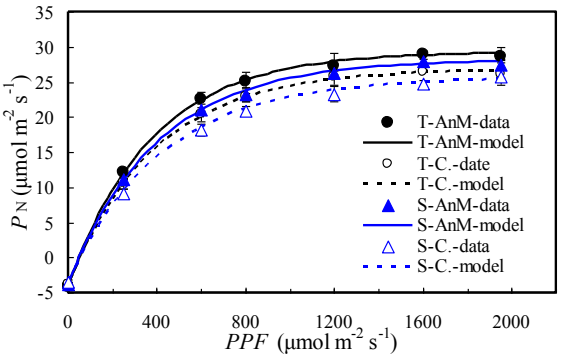

Fig. 6.3 Leaf photosynthetic light response curves for peanut plants. data, the average of original data (n=12); model, the models analyzed by $P_N = P_C(1\text{-}e^{-KI}) - R_D$ according to the original data. T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-C., transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-C., direct seeding without hypocotyl exposing treatment. Error bars represent SE values for twelve independent replicates.

6.4.5 Seedling transplanting induced osmotic adjustment

As shown in Table 6.4, Ψ_{FT} was the leaf water potential (Ψ) when leaf water was saturated. Ψ_{FT} showed no significant difference among treatments. The osmotic potential (π) in the symplastic solution theoretically diluted by apoplastic water (π_{s+a}) was lower in seedling transplanted crops and AnM treated crops than in their controls. The osmotic potential at full turgor (π_{FT}) was significantly lowered by seedling transplanting and more obviously minus than AnM treatment. Lower π meant higher osmotic concentration (C_{FT}) in the cells. With the plot of direct seeding without AnM treatment as the zero reference, the net active solute accumulation (ΔC_{FT}) caused by osmotic adjustment was calculated and confirmed that extra osmotic adjustment was really induced by seedling transplanting and AnM treatments. The values of π and ζ at incipient plasmolysis or zero turgor (π_{IP} and ζ_{IP}) were lower in seedling transplanted crops and AnM treated crops than in their controls. This suggested that leaves of peanut plants in plots of seedling transplanting and AnM treatments could maintain turgor potential until a lower π and a lower ζ compared with their controls. A lower π_{IP} or ζ_{IP} means a higher drought tolerance. It was suggested that both seedling transplanting and AnM treatment increased drought tolerance, if any, for the peanut crops. As compared with AnM practice, seedling transplanting showed the same effect as AnM method to improve cell water compartment by enlarging the symplastic water fraction (ζ_{sym}) which favorites biochemical functions and cell turgor maintenance, especially in case of water deficit. At midday when evaporation would be the highest in the day and stomata will close to reduce water loss, the cell water condition would be limited. In the present experiment, seedling transplanting lowered the midday osmotic potential (π_{MD}) with significant synergistic interaction with AnM method. The high π_{MD} resulted in high midday turgor potential (P_{MD}) which indicated that seedling transplanting with the AnM method increased the plant resistance to environment changes by hypocotyl exposure.

Table 6.4 Parameters of pressure-volume curves for peanut plants.

Treat.	Ψ_{FT}	π_{FT}	P_{FT}	π_{s+a}	π_{IP}	Ψ_{MD}	π_{MD}	P_{MD}	α	β	ζ_{IP}	ζ_{sym}	ζ_{apo}	C_{FT}	ΔC_{FT}
	----------(MPa)----------								-(×10^{-3})-					-(osmol m^{-3})-	
T-AnM	-0.21	-1.61	1.40	-1.12	-1.850	-1.18	-1.64	0.46	55.2	0.98	0.868	0.736	0.264	660.1	77.9
T-Control	-0.21	-1.52	1.31	-1.02	-1.725	-1.19	-1.57	0.38	49.3	0.97	0.882	0.706	0.294	623.2	41.0
S-AnM	-0.20	-1.49	1.29	-0.97	-1.620	-1.17	-1.59	0.42	52.3	0.97	0.865	0.733	0.267	610.9	28.7
S-Control	-0.20	-1.42	1.22	-0.91	-1.571	-1.18	-1.45	0.27	47.9	0.95	0.871	0.712	0.298	582.2	0.0
Transplant	ns	**	*	*	**	ns	*	**	*	*	ns	ns	ns	*	**
AnM	ns	*	*	*	*	ns	*	**	**	*	*	**	**	**	**
T×AnM	ns	*	ns	ns	**	ns	*	*	*	*	*	*	ns	ns	**

T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. The P-V curve was modeled as follow,

$-\Psi^{-1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]$.

Ψ_{FT}, leaf water potential at full turgor or in saturated leaf water condition; Ψ_{MD}, leaf water potential at midday; π_{FT}, osmotic potential at full turgor; π_{MD}, osmotic potential at midday; π_{s+a}, the osmotic potential in the symplastic solution theoretically diluted by apoplastic water; π_{IP}, osmotic potential at incipient plasmolysis; P_{FT}, leaf turgor potential at full turgor; P_{FT}, leaf turgor potential at midday; P_{MD}, leaf turgor potential at midday; ζ_{apo}, apoplastic water fraction shown as $\zeta_{apo} = \zeta_{FT}-\zeta_{sym} = (1-\zeta_{sym})$, where, ζ_{sym} is the symplastic water fraction; ζ_{IP}, relative leaf water potential at incipient plasmolysis; β and α, constants; C_{FT}, osmotic concentration at full turgor; ΔC_{FT}, increment of osmotic concentration caused by active osmotic adjustment compared with the control.

*, ** and ns mean significance at $P\leq0.05$, at $P\leq0.01$ and no significance according to LSD analysis.

6.4.6 Seedling transplanting with AnM increased water retention ability

Water retention ability was estimated by analyzing the excised leaf transpiration declining curve. The relative water content when stomata closed (ζ_{SC}) was lower in T-AnM treatment than in their controls (Table 6.5). In other words, leaves in seedling transplanted AnM treatment could open their stomata to lower levels of relative water content. This suggested that physiological activities such as stomatal conductance in seedling transplanted peanut plants could be more tolerant to water deficit compared with the control. The time of stomatal closure (t_{SC}) was shortened by AnM treatment (T-AnM and S-AnM), but showed slightly shorter in seedling transplanted plants (T-Control) than in S-Control. This suggested that the stomata were more sensitive to the leaf water loss in seedling transplanted plants and AnM treated plants than in their controls. The point (t_{SC}, ζ_{SC}) was defined as stomata closure point and it was adjusted by the time constant α, which was larger seedling transplanted peanut plants and AnM treated plants than the controls. The time (τ_D) used to dry the excised leaf to a relative water content of 10% was prolonged by seedling transplanting and AnM. It was adjusted by the constant β, which related to cuticular transpiration and was smaller in seedling transplanted peanut plants and AnM treated plants than the controls.

Table 6.5 Parameters from the analysis of excised leaf transpiration declining curves.

Treatment	ζsc	t_{SC} (min)	α	β	τ_D (min)
T-AnM	0.782	26.2	0.0924	0.00104	839
T-Control	0.805	36.3	0.0798	0.00122	718
S-AnM	0.796	27.5	0.0802	0.00113	774
S-Control	0.793	38.2	0.0627	0.00129	680
Transplant	*	ns	*	*	**
AnM	*	**	**	**	**
T×AnM	*	**	*	*	*

T-AnM, transplanting of seedlings with elongated hypocotyl to be exposed to light and dry air; T-Control, transplanting of seedlings with un-elongated hypocotyls; S-AnM (modified AnM), direct seeding with hypocotyls exposed to light and dry air by removing the cover soils; S-Control, direct seeding without hypocotyl exposing treatment. The curve was modeled as $\zeta=[\zeta_{sat}-\zeta_{SC}(1-\beta t)]e^{-\alpha t}+\zeta_{SC}(1-\beta t)$. ζ_{SC}, the leaf relative water content at the time when the stomata closed; β, a constant related to cuticular transpiration; α, a constant related to the stomatal transpiration; t_{SC}, time when stomata started to close; τ_D, the time used to dry the excised leaf to a relative water content of 0.10. *, ** mean significance at $P\leq0.05$, at $P\leq0.01$ according to LSD analysis.

6.5 Discussion

In the present experiment, extra elongation of hypocotyls was induced and exposed to light but it was not the real AnM practice in the field. Nevertheless, using seedlings with extra elongated hypocotyls increased plant biomass and shell yield by 12.11% and 7.95% respectively, compared to those in modified AnM only. Transplanting seedlings with un-elongated hypocotyl did not significantly increase plant biomass but did significantly increased pod biomass and shell yield. The additive and/or synergistic effects of seedling transplant in combination with AnM practice in increasing plant biomass, pod biomass and shell yield reached 23.7%, 33.3% and 33.6% respectively. AnM treatments including the hypocotyl exposure of the seedlings increased pod number but the pod size was lower that their control. Plants in control plots tried to compensate the shell yield loss with low pod number by increasing the pod size. Both seedling transplanting and AnM treatments slightly increased harvest index. Because extra elongation was intentionally induced, the hypocotyl was much longer in AnM treatments than in control plots. Both results from direct seeding and transplanting of seedlings with elongated and exposed hypocotyls demonstrated the profound positive effect of suggested hypocotyl exposing treatment on peanut crops. When designing the experiment, we just intended to use the seedlings for easy and convenient treatment of the hypocotyl exposure but did not expect its yield increasing effect. Fortunately, seedling transplanting showed innegligible yield increasing effect in peanut crops.

Seedling transplanting is adopted in peanut production to solve the problems of bad seed germination and seedling establishment in spring when the soil temperature is low and the delay of seeding for summer rotated peanut crops after wheat harvest (Wang et al. 2002). The establishment of the summer peanut crop can be 15 day advanced and growth period is prolonged by seedling transplanting. The yield increasing effect by seedling transplanting is reported up to 12.6% to 22.2% (Wang et al. 2002). In the present study, the pod yield increases by seedling transplanting and AnM treatments was attributed to increases in the whole plant growth, which might be attributed to improvement in photosynthetic activity on the increased biomass basis. We used a modified sigmoid curve equation to analyze the growth dynamics during the whole growth period. Results of the growth dynamic analysis of plant and pod growth and supported the data of yield components and showed the growth advantages of transplanted plants over the directly seeded plants during the whole growth period and also showed growth advantages of AnM treated plants over the control plants during the later growth period (Fig. 6.2). Increases in plant biomass production were attributed to leaf photosynthetic activity on the improved biomass basis that was proportional to leaf area. Both photosynthetic capacity and quantum use efficiency were improved by seedling transplanting and AnM treatment. Improvements in photosynthesis might result from leaf turgor improvement, which was in turn attributed to osmotic adjustment. We used a modified P-V curve equation to estimate osmotic and cell water

compartment. Results showed that leaf osmotic potential was lower but the leaf water potential was the same and therefore the leaf turgor potential was maintained higher in seedling transplanted AnM treatment. The P-V curve analysis also showed that the water fraction in the symplasm was higher or the water fraction in the apoplasm was lower in seedling transplanted plants and AnM treated crops than in their controls. As speculated, in response to the stimuli of transplanting or AnM treatment, the plant cell re-compartmented its water content by moving some of the water in apoplasm (cell walls) into the symplasm, where most of the metabolism occurred. More symplastic water fraction would favorite metabolisms in the symplasm and this might be one of the xerophytophysiological regulations (Patakas and Noitsakis 1997). Several reports from my previous experiments have proved that AnM technique induces xerophytophysiological regulations such as osmotic adjustment and increasing symplastic water fraction and consequently improves plant growth, photosynthetic activities and final shell yield. In addition to the confirmation of the AnM effect, results of the present experiment demonstrated the xerophytophysiological regulations and the consequent improvements in plant growth, leaf photosynthesis and yield caused by seedling transplanting in peanut crops. As reported by Xu et al. (2011c), xerophytophysiological regulations such as osmotic adjustment and increasing symplastic water fraction were induced by transplanting wheat seedlings, which were air dried for a moment to experience a stimulus of drought stress. The transplanted seedlings produced a healthy wheat crop with improved plant growth and leaf photosynthesis and increased grain yield. In the present experiment, the peanut seedlings intentionally dried but the seedlings already reached half-wilting status before transplanted into soils because the temperature and light intensity were high and air humidity was low, which suggested that the peanut seedlings had experienced a stimulus of drought stress in addition to transplanting hurt. That was why seedling transplanting showed a xerophytophysiological effect similar to the AnM treatment.

In addition to the strengthening physiologically by osmotic adjustment, morphological strengthening was also induced by seedling transplanting and AnM treatments. In the field, it was observed that leaves deep in green and plant stands were stronger in seedling transplanted and AnM treated plants. In the present study, we used the excised leaf transpiration declining curve to analyze the leaf water retention ability. Results showed that water was less lost through cuticular transpiration in the excised leaves from the seedling transplanted and AnM treated plants, which suggested the cell epicuticular layer thickening and more wax depositing onto the leaf surface were induced as one of the xerophytophysiological regulations (Premachandra et al. 1992; Xu 2007). Since the cuticular transpiration water loss was lower, the time used to dry the excised leaf to 10% of relative water content, i.e., the air dry status (τ_D) was longer for seedling transplanted peanut plants and AnM treated plants. The strengthening of the leaf epicuticular structure might also contribute to the disease resistance as above

described (Table 6.1). In previous experiments, it was already found that peanut plants grown with AnM techniques showed lower cuticular transpiration water loss and in the present experiment this was once more confirmed. Not only the AnM treatments but also the seedling transplanting lowered the cuticular transpiration water loss as a consequence of xerophytophysiological regulation. This suggested that seedling transplanting could be used as a xerophytophysiological treatment as reported in case of transplanted wheat crops (Xu et al. 2011c). Both analyses of P-V curve and leaf transpiration declining curve suggested that seedling transplanted and AnM treated peanut plants were more resistant to dehydration that would be caused by either soil water deficit, low humidity, high irradiation, or rhizosphere salinity.

Morphological strengthening of the plants might also contribute to the disease resistance. In the present study, the severity index of brown leaf spot was lower in seedling transplanted and AnM treated plants than their control. Agronomically, AnM techniques are used to lift up the cotyledon node out of the soil surface so that early seed setting was controlled from the first two branches from the cotyledon node. When the cotyledon node remains inside the soil, the early pods will compete for nutrition and carbohydrates with the whole plant of the young seedling and rot in soil causing aflatoxin contamination to later pods. In the present study, rot pods were fewer in seedling transplanted and AnM treated plants. It has already been found that AnM techniques prevent formation of early pods by controlling early flowering and early penetration of the pegs. The present experiment confirmed results of my previous experiments. At the beginning, AnM practices or clearing soil around was used to solve the problem of too early formation of pods, which might be contaminated by aflatoxin and further pollute the shell product and fewer lower the product quality (Shen 1958; Shen and An 1988; Chemberlin et al. 2004; Craufurd et al. 2006). In addition to the positive effect of AnM treatment, seedling transplanting also proved effective in prevention of early pods and rot pods. Although research and production all have proved that AnM techniques improve plant growth and productivity as well as resistances to diseases and environmental stresses in peanut crops (Shen and An 1988), practices of AnM techniques are labor-cost and unfeasible for mechanization. Moreover, peanut seedlings can be raised in large scales in artificial facilities, where extra-elongation can be easily induced in dark conditions and exposed to designed lighting and dry air as stimuli to induce xerophytophysiological regulations and the consequent health of the crop. The seedling raising practices in the present experiment proved that raising healthy seedlings in scales is feasible. In China, trials have proved that the mechanization of seedling transplanting is also possible because a peanut seedling transplanter is also available as a rice seedling transplanter.

In conclusions, transplanting of seedlings with extra elongated hypocotyls, which were exposed to light and dry air for a period, improved plant growth, leaf photosynthesis and shell yield of peanut crops. It could be used as an alternative of

hypocotyl exposure in the field since the practices of hypocotyl exposure in AnM techniques are complicated and labor cost. Similar to the effect of hypocotyl exposure in AnM techniques, the effect of seedling transplanting was also mainly based on stimulation-induced osmotic adjustment which improved leaf turgor maintenance and consequently improved plant growth, leaf photosynthesis and shell yield of the peanut crops. Therefore, seedling transplanting could also be considered as one of stimulation treatments to induce xerophytophysiological regulations in addition to its agronomical merits such as advancing the plant establishment and prolonging the growth period.

Chapter 7

Hypocotyl Exposing Treatment Reduces Photosynthetic Hysteresis in Peanut Plants

7.1 Abstract

When photosynthetic photon flux (*PPF*) is changed from low to high and then from high to low, the photosynthetic rate (P_N) at each *PPF* level is usually higher during the cycle of changing *PPF* from high to low than during the cycle from low to high. The difference between the two *PPF* response curves is defined as photosynthetic hysteresis (H_P). H_P would be smaller if physiological activities are high in a plant. The previous experiments of our research have demonstrated that physiological activities are higher in peanut plants grown with the AnM technique including hypocotyl exposure. In the present experiment, H_P was examined in the peanut crop grown with the AnM technique. Peanut leaf photosynthetic activities were improved by exposing the hypocotyl, one practice of the AnM technique, and film mulching, the combined practice of the modified AnM technique. The improved photosynthetic activities also reflected in less H_P. H_P was consistent with hysteresis of stomatal and mesophyll conductance. Less H_P could be an indicator of higher physiological activities, as improved by the hypocotyl exposing and film mulching in the peanut crop.

***Key words*:** hysteresis, hypocotyl exposure, peanut (*Arachis hypogaea* L.), photosynthetic capacity, mathematics, mesophyll conductance, stomatal conductance.

7.2 Introduction

The term of hysteresis in physics, meaning "deficiency" or "lagging behind" (Wikipedia, the free encyclopedia), represents a retardation of the effect when the forces acting upon a body are changed. A system with hysteresis exhibits independent irreversible behavior, or "rate-independent memory" (Poulovassilis 1962; Mielke and Roubicek 2003), distinguishing hysteresis from most other dynamic processes in many systems. Hysteresis is a typical class of strongly nonlinear natural phenomena in physical fields. It is described as "when there are two quantities of y and x, such that cyclic variations of x cause cyclic variation of y, then if the changes of y lag behind those of x, it can be said there is hysteresis in relation of y and x" (Ewing 1885). The curve for decreasing x does not coincide with that for increasing x (Poulovassilis 1962; Xu 2000; Flynn et al. 2003; Xu et al. 2009a). The relationship between y and x is not unique and irreversible. The asymmetric response of photosynthetic entity has been termed the 'hysteresis effect', since there is a lag in the process in relation to its driving force. For example, in magnetic (Ewing 1885; Tauxe et al. 1996), ferromagnetic (He and Wang 1993), elastic (Muller and Xu 1991) and electric (Bassiouny et al. 1988) behaviors of materials, a lag occurs between the application and the removal of a force or field and its subsequent effect. Hysteresis also happens in lots of other scientific fields, such as economics, electrics and biology (Flynn et al. 2003). In cell biology, cell division exhibits hysteresis in which it takes a higher concentration of cyclins to switch cells from G_2 phase into mitosis than to stay in mitosis once begun (Pomerening et al. 2003). In crop scientific field, hysteresis could occur in the response of photosynthesis to irradiance. This natural phenomenon of photosynthetic hysteresis occurs universally in phytoplankton (Falkowshi and Owens 1978), algae (Han et al. 2000) and higher plants, i.e., scots pine (Ng and Jarvis 1980), rice (Bios et al. 1985), soybean (Wang et al. 2007), microphytobenthos (Serôdio et al. 2008) and so on. These studies are noticed on diurnal hysteresis that photosynthetic performance was lower in the afternoon than in the morning at the same light intensities. This hysteresis phenomenon is often called the "afternoon depression" of photosynthesis. Several hypotheses for the mechanisms of the diurnal hysteresis include, 1) diurnal changes in photosynthetic activity due to circadian rhythm (Post et al. 1985); 2) diurnal changes in nutrient and CO_2 availability; 3) decrease in photosynthesis due to photoinhibition after long exposure to high light intensities; 4) an increase in photorespiration in the afternoon (Falkowski et al. 1985); and 5) photosynthetic feedback inhibition in the afternoon (Wang et al. 2007). In contrast to all previously published cases, research from Levy et al. (2004) revealed an unexpected hysteresis called "morning depression" with higher photosynthetic rates occurring in the afternoon than in the morning. Their interpretation is that following a prolonged exposure to darkness, the photosynthetic machinery is incapable of utilizing intense photon flux as efficiently as it does later in the day. In addition of diurnal hysteresis that happened after a long period exposure to irradiation, Xu and his research

group found photosynthetic hysteresis could also occur within short time, i.e. 15-30 min, when photosynthetic photon flux (*PPF*) is changed from low to high and then from high to low (Xu et al. 1994; Xu 2000; Xu et al. 2009a). Photosynthetic rate is usually higher during the second cycle of *PPF* changes than the first one (Ng and Jarvis 1980; Jones et al. 1984; Xu et al. 1994; Xu 2000) even from high *PPF* to low and then reversed from low to high (Xu et al. 1994). These papers presented the mechanism of photosynthetic hysteresis, which based on the fact that sufficient time is needed for stomata to get fully open and for photosynthetic apparatus to get completely activated (Burrow and Milthorpe 1976; Warrit et al. 1980; Xu et al. 1994). Hysteresis occurs especially when there is no enough time allows for completion of equilibrium at one *PPF* point (Xu 2000). Actually, photosynthetic hysteresis is an adaptability of plant to unexpected disturbances in the environment. It is usually happened in the senescent or stressed leaves, where stomata are more difficult to open fully than those in normal plants in response to *PPF* changes (Xu et al. 1994; Xu 2000). Hypocotyl exposed by removing soil away around the base part of the peanut seedling has proved effective in improvements of leaf turgor, photosynthesis, plant growth and shell yield in peanut crops. This treatment at seedling stage is actually based on the theory of signal transduction and xerophytophysiology (Xu 2007), giving a false signal of water deficit to the plants, inducing internal adjustment from the gene level to physiological levels, including osmotic adjustment, maintenance of a high leaf turgor potential and a high symplastic water fraction, morphological strengthening as well as photosynthesis improvement. As far as we know, in contrast to the well documented “diurnal hysteresis”, photosynthetic hysteresis occurring in short time has not been well described. In the present study, the further study of photosynthesis affected by hypocotyl exposure was examined in peanut plant.

7.3 Materials and Methods

7.3.1 Plant material and treatment

In order to estimate how hypocotyl exposure in the techniques of AnM affected photosynthesis, a 2×2 factorial experiments was designed with film mulching as the main plot and AnM technique as the sub-plot. Thus, 4 treatments were as follows: 1) control, no-mulching without AnM; 2) basic AnM; 3) mulching; and 4) modified AnM. Seeds of peanut (*Arachis hypogaea* L. cv. Chibahandachi) were sown in a ridge in the middle of May. The main objective of the present study was to investigate the sensitivity of leaf photosynthesis to light irradiation affected by hypocotyl exposure. Therefore, the investigation stopped at the "n" stage and the hypocotyl exposure treatment lasted for two weeks. Basic and modified AnM were referred to Chapter 2 and Chapter 3.

7.3.2 Leaf photosynthesis, stomatal and mesophyll conductances

Analysis of leaf photosynthesis was the same as described in Materials and Methods 2.3.3 in Chapter 2.

Stomatal conductance for vapor, intracellular CO_2 concentration (C_i) and transpiration rate (T_r) were simultaneously recorded by the same LI-6400 portable photosynthesis system (LI-COR, Co., Ltd., Lincoln, Nebraska, USA). Mesophyll conductance (g_M) was calculated from P_N and C_i as P_N/C_i (Jones et al. 1984). The stomatal conductance (g_S) to CO_2 into a leaf was calculated as stomatal conductance to vapor multiplied by a coefficient of 0.62 because of the lighter molecular mass and faster diffusion of H_2O relative to those of CO_2. The light response curve for g_S was analyzed as $g_S = g_{S\text{-}C}\ (1\text{-}e^{-KI})\text{-}g_{S\text{-}O}$, and g_M was analyzed as $g_M = g_{M\text{-}C}\ (1\text{-}e^{-KI})\text{-}g_{M\text{-}O}$. In both equations the subscripts C means the capacity and O means the g_S or g_M before light on.

7.3.3 Photosynthetic hysteresis

The photosynthetic light response curve is modeled as $P_N = P_C(1\text{-}e^{-KI})\text{-}R_D$. The difference between the two curves was defined as photosynthetic hysteresis (H_P). The extent of hysteresis (H_P) was calculated by $H_P = 1\text{-}\int_a^b f(I_L) \Big/ \int_a^b f(I_H)$, where, the I_L and I_H were the photosynthetic photon flux (*PPF*) which was started from low to high and from high to low, respectively; H_P was the relative value of the area surrounded by the two photosynthetic light response curves within the range from a (0) to b (2000). The hysteresis of stomatal conductance (H_S) and mesophyll conductance (H_M) were calculated the same. Fig. 7.1 shows a sample curve for photosynthetic hysteresis.

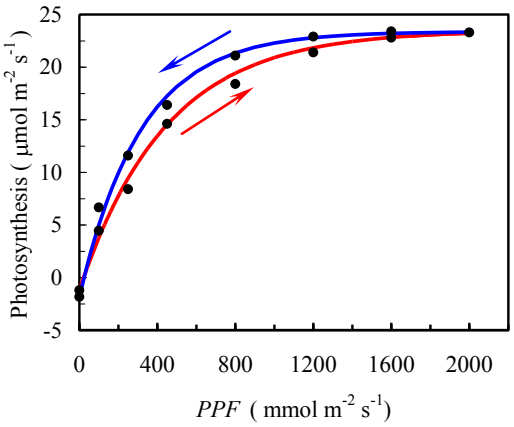

Fig. 7.1 A model curve for photosynthetic hysteresis. The red curve indicates photosynthetic light response curve within the range of *PPF* increase from 0 to 2000; the blue curve indicates photosynthetic light response curve within the range when *PPF* decreases from 2000 to 0.

7.4 Results

7.4.1 Hypocotyl exposure improved photosynthesis, stomatal and mesophyll conductance

The main objective of the present study was to investigate the sensitivity of leaf photosynthesis to light irradiation affected by hypocotyl exposure. Therefore, the investigation stopped at the "n" stage after hypocotyl exposure treatment lasting for two weeks. As shown the same results in the previous experiments, hypocotyl exposure treatment in both the basic and modified AnM improved the leaf photosynthetic activities including the high photosynthetic rate (P_N), photosynthetic capacity (P_C), maximum quantum yield (Y_Q) and compensation point (PPF_C) (Fig. 7.2 A; Table 7.1). This result was also found in stomatal conductance (g_S) and mesophyll conductance (g_M), suggesting that exposing hypocotyls to sunlight and dry air at peanut seedling stage had some potential benefits to leaf photosynthesis.

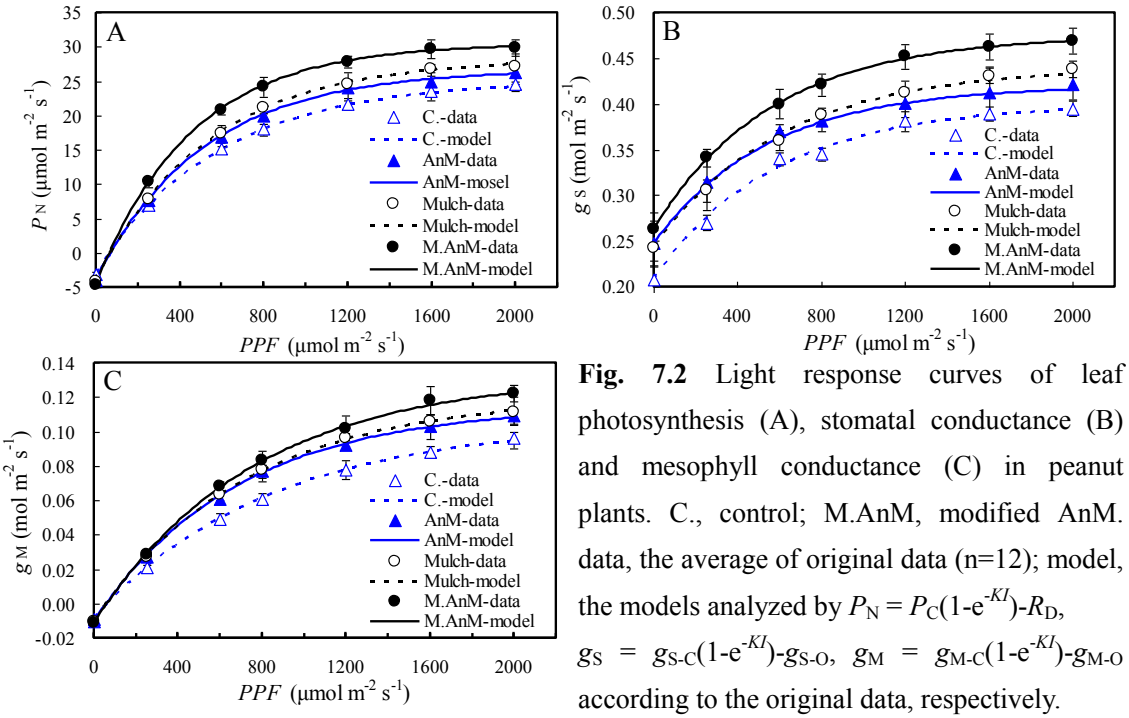

Fig. 7.2 Light response curves of leaf photosynthesis (A), stomatal conductance (B) and mesophyll conductance (C) in peanut plants. C., control; M.AnM, modified AnM. data, the average of original data (n=12); model, the models analyzed by $P_N = P_C(1-e^{-KI})-R_D$, $g_S = g_{S\text{-}C}(1-e^{-KI})-g_{S\text{-}O}$, $g_M = g_{M\text{-}C}(1-e^{-KI})-g_{M\text{-}O}$ according to the original data, respectively.

Table 7.1 Parameters from the light response curves of leaf photosynthesis (P_N), stomatal conductance (g_S) and mesophyll conductance (g_M) in peanut plants.

Treatment	Leaf photosynthesis				
	P_C	R_D	K	Y_Q	PPF_C
	-(μmol m^{-2} s^{-1})-		(m^2 s μmol^{-1})	(mol mol^{-1})	(μmol m^{-2} s^{-1})
Control	28.2	3.07	1.71	48.2	67.5
Basic AnM	31.6	3.89	1.93	61.0	70.5
Mulching	32.4	4.15	1.86	60.3	71.7
Modified AnM	35.1	4.61	2.19	76.9	68.3
AnM	**	*	*	**	ns
Mulching	**	*	*	**	ns
A×M	*	*	*	*	ns

	g_S			g_M		
	$g_{S\text{-}C}$	$g_{S\text{-}O}$	K_S	$g_{M\text{-}C}$	$g_{M\text{-}O}$	K_M
	-(mol m^{-2} s^{-1})-		(m^2 s mol^{-1})	----(mol m^{-2} s^{-1})----		(m^2 s mol^{-1})
Control	0.401	0.208	1.71	0.114	0.009	1.19
Basic AnM	0.421	0.247	1.83	0.125	0.010	1.46
Mulching	0.442	0.242	1.64	0.131	0.011	1.29
Modified AnM	0.472	0.263	1.76	0.144	0.012	1.42
AnM	**	*	*	*	ns	**
Mulching	**	*	ns	**	*	ns
A×M	*	*	*	*	*	*

P_C, photosynthetic capacity; R_D, respiration rate; K, the half time constant for photosynthetic light response curve, equivalent to the reciprocal of the PPF at which P_N reaches 63 % of P_C; Y_Q, the maximum quantum yield defined as $Y_Q = KP_C$; PPF_C, compensation point defined as PPF at P_N=0. $g_{S\text{-}C}$ and $g_{M\text{-}C}$, the capacity of g_S and g_M, respectively; $g_{S\text{-}O}$ and $g_{M\text{-}O}$, the g_S or g_M before light on. *, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=3).

7.4.2 Hypocotyl exposure decreased photosynthetic hysteresis

Hysteresis of all of P_N, g_S and g_M was smaller in peanut plants with hypocotyls exposing treatment than in the un-exposed control and also smaller in mulched plots than in un-mulched control no matter with or without hypocotyl exposing treatment (Table 7.2). The results suggested hypocotyl exposing treatment and film mulching increased physiological activities of photosynthetic machinery including the related enzymes in leaves of peanut plants.

Table 7.2 Hysteresis of leaf photosynthesis (H_P), stomatal conductance (H_S) and mesophyll conductance (H_M) in peanut plants.

Treatment	H_P	H_S	H_M
Control	0.033	0.13	0.078
Basic AnM	0.015	0.06	0.051
Mulching	0.025	0.15	0.066
Modified AnM	0.012	0.08	0.034
AnM	**	**	**
Mulch	*	*	**
A×M	*	*	*

*, ** and ns mean significance at $P \leq 0.05$, at $P \leq 0.01$ and no significance according to LSD analysis (n=3).

7.5 Discussion

In the previous experiments, a hypothesis was proposed that hypocotyl exposure would be the key for both AnM and modified AnM method. In addition to the original propose of preventing the early penetration of pegs, it belongs to one of the effective practices of xerophytophysiological applications (Xu 2007). Usually, the hypocotyls of peanut seedlings grow underground or covered over by surface soil. Hypocotyls function to some degree as roots to absorb water and nutrition from soil. Adventitious roots develop from the base part of hypocotyl. As exposed by removing soil away, the fleshy hypocotyl experiences the unexpected changes of environment, i.e., the sudden light irradiation from underground darkness, the relatively low humidity or the higher temperature due to the irradiation or some other changes. However, the roots still grow underground and the irrigation is normal. There is no the so-called real water stress but only moderate stimuli by irradiation and low air humidity. These environment changes caused by hypocotyl exposure could give the seedlings some signals or stimuli that seedlings adjust their internal changes in response and adaption to the environment changes (Xu et al. 2009a). This might be one of the reasons for the higher photosynthesis in hypocotyl exposed seedlings.

When suddenly imposing a light radiation to the leaf, the stomata start to open and the related enzymes are activated in response to the irradiation. Plants with higher physiological activities and biochemical activities and/or without stresses are supposed to respond the light imposing faster. If the activities are not high or there are some kinds of stresses or inactivation caused by stresses, the plants would respond to the light slower and could not make the photosynthesis to reach its potential at that light intensity before the light intensity is changed to a light level. So the photosynthetic rate lower than what it can be, if the light imposition is prolonged enough, is recorded. When the light is changed from high to low, the stomata and photosynthetic machinery including the related enzymes have been activated by the first cycle of illuminating and therefore P_N at each light intensity point is higher than at the same light point during the first cycle of illuminating, making a hysteresis of P_N between the two cycles of measurement. That is why the hysteresis would be large if the physiological and biochemical activities were low. The hysteresis of g_S might be related with the related physiological activities and that of g_M might be related with the biochemical and morphological factors, which are reflected in several diffusive conductances for CO_2 along the gradient from air to inside space of mesophyll cells, where CO_2 is fixed by rubisco, the key enzyme in the photosynthetic process (Nobel 1999; Evans and Loreto 2000). The main diffusive conductances are stomatal conductance and mesophyll conductance. Stomatal conductance is the speed at which CO_2 is taken in from the air into the leaf mesophyll and water transpires from pores in a plant to the air, and is directly related to relative size of the stomatal aperture. Basically, the higher the transpiration rate, the higher the conductance of the leaf. Air humidity, the water status

of the plant, plant hormone relation, light intensity and much more other factors also affect stomatal conductance. When plants are moved from dark to light, stomata open in response to light. The opening and closing of a stoma is controlled by guard cells, a pair of crescent-shaped cells that surround the pore opening. A stoma opens when guard cells absorb water, and closes when they lose water. When the guard cells become turgid, the stomata open and the stomata close when guard cells become flaccid. When light, especially the blue light, hits the stomata it causes the hydrogen channels to open. Hydrogen is pumped in response to increased blue light levels. In response to hydrogen moving out potassium moves in, and water follows, and turgor pressure of guard cells increases (Farquhar and Sharkey 1982). The process is related with a series of physiological and biochemical (if the hormone abscisic acid is involved) activities. Another is mesophyll conductance (g_M), from intercellular spaces to the site of fixation, which pose a significant limitation to photosynthesis (De Lucia et al. 2003; Galle et al. 2009). Study by Warren et al. (2003) indicates that g_M limits photosynthesis by the same magnitude as g_S does. In present experiment, hysteresis of g_S and g_M was consistent with P_N hysteresis and also consistent with magnitude of P_N. This suggested that both g_S and g_M contributed to P_N and P_N hysteresis was attributed to hysteresis of both g_S and g_M. The results of the present experiment suggested that less hysteresis of P_N as well as that of g_S and g_M in the peanut plants with hypocotyl exposing treatment or film mulching was consequence of the improved physiological activities including the improved photosynthetic activities as well as leaf improvement by osmotic adjustment as reported in the previous research.

In conclusion, photosynthetic activities shown as photosynthetic capacity and the maximum quantum use efficiency in peanut plants were improved by exposing the hypocotyl exposure, one practice of the AnM and modified AnM techniques in peanut production. The improved photosynthetic activities also reflected in less hysteresis of photosynthesis. Photosynthetic hysteresis was consistent with hysteresis of stomatal and mesophyll conductance. It was concluded that less hysteresis of photosynthesis as well as stomatal and mesophyll could be an indicator of physiological activities, as consequences of improved physiological activities by the hypocotyl exposure the peanut crop.

Chapter 8

Stomatal Oscillations in Intact and Excised Leaves of Peanut Plants grown with A Modified AnM Technique

8.1 Abstract

Previous research has shown that AnM and the modified AnM (AnM combined with film mulching) techniques improve photosynthesis, plant growth and the final shell yield in peanut plant. Improvement in net photosynthetic rate (P_N) was attributed at least in part to increases in stomatal conductance (g_S). Moreover, g_S often oscillates in response to environmental changes. Oscillations also occur in the excised leaf during the declining of leaf transpiration. Results of analysis showed that stomata oscillated with higher amplitude and shorter period in leaf of AnM treated peanut than in control. During the transpiration declining in the excised leaves, oscillations of g_S or P_N were found in AnM treated peanut plant but were not clear in control. The water retention ability was higher in the excised leaves from AnM treated plant than those from control. In conclusion, peanut plants grown with the modified AnM technique could prevent leaf water loss by controlling the stomatal opening and closing regularly.

Keywords: AnM method, mathematical modeling, oscillation, peanut (*Arachis hypogaea*), photosynthesis, stomata, transpiration, water deficit, xerophytophysiology.

8.2 Introduction

The term “oscillation” is the repetitive variation, typically in time, of some measure about a central value or between two or more different states. Familiar examples in physics include swinging pendulum and AC power. The term vibration is sometimes used more narrowly to mean a mechanical oscillation but sometimes is used to be synonymous with “oscillation”. Oscillations occur not only in physical systems but also in biological systems. Photosynthetic oscillation is an abnormal photosynthetic response phenomenon that photosynthetic rate declines for a while, recovers to some extent and drops again, showing cyclic changes (Cowan 1972ab; Barrs 1987; Hirose et al. 1991; Xu et al. 1994). Cyclic changes in photosynthetic rate have been observed in many plant species, such as cotton (Eherler et al. 1965), soybeen (Chen et al. 1971), rice (Tanaka 1978), oats (Johnson et al. 1979), orange (Levy and Kaufmann 1976), rose (Rose 1994), tomato (Xu et al. 1994) and peanut (Hirose et al. 1991). Oscillation is an adaptation mechanism against adverse environmental conditions. It is reported that photosynthetic oscillation is caused by stomatal response. Stomata control CO_2 absorption for photosynthesis and water vapor release through transpiration, functioning as the gate between the plant and the environment. There are several hypotheses for the mechanism of stomata oscillation. For example, suddenly changing irradiation or moving the plants from dark to high PPF, can induce photosynthetic oscillation (Hirose et al. 1994). It is the dynamics of water balance in response to changes in leaf water status. In the case of a very high evaporation demand oscillation occurs to adjust the balance between water uptake and transpirational water loss (Farquhar and Sharkey 1982; Bunce 1987). When water uptake cannot balance the transpired water loss, leaf tissue loses its water and subsequently leaf turgor is reduced, and stotama close. Transpiration decreases when stomata close and water uptake surpasses transpiration water loss and leaf tissue stores excess water. When the stored water is much enough to rehydrate the leaf, stomata open again. It seems that a mechanism like capacitance is involved in leaf water influx and efflux.

Stomata in peanut leaves are sensitive to environment changes and oscillations of stomatal conductance are often observed in peanut leaves in the field when the evapotranspiration demand was high (Hirose et al. 1991; 1992). Stomatal oscillation, which is defined as a phenomenon of cyclic opening and closing movement of stomata (Barrs 1971), and its consequent oscillations in photosynthesis are responses of plants to environmental changes and related with the physiological activities in the plants. Therefore, in the present experiment, oscillation of stomata conductance and the consequence oscillation of photosynthetic rate were confirmed in intact leaves and in the excised leaves of peanut plants. The effects of the modified AnM technique on the stomatal oscillations were clarified. Mathematic equations were used to analyze the dynamics of the oscillations of stomatal conductance and photosynthetic rate.

8.3 Materials and Methods

8.3.1 Plant materials and treatments

Seeds of peanut (*Arachis hypogaea* L. cv. Chibahandachi) were sown in early May in PEP rainout shelters. The soil belonged to Andosol, a volcano ash, with the properties characterized by pH, 6.0; EC, 0.05 mS cm^{-1}; total C and total N 38.3 and 3.1 g kg^{-1}, respectively; NH_4-N, NO_3-N, P, K, Ca and Mg, 6.4, 5.2, 169.6, 346.3, 2632.0 and 438.0 g kg^{-1}, respectively; and CEC (cation exchange capacity), 18.6 cmol kg^{-1}. A biofertilizer fermented using rice bran, soil mill sludge and fish meal as materials was applied 210 g m^{-2} on dry mass basis in late April, two weeks before sowing. The biofertilizer contained N, P, and K of 40.2, 28.3 and 19.2 g kg^{-1}, respectively. The peanut plants were irrigated properly according to the evapotranspiration demand by a spray system over the crop and under the roof of the rainout shelter. Two treatments were designed as the modified AnM practice and control. The modified AnM practice referred to Chapter 3.

8.3.2 Measurements of leaf photosynthesis and stomatal conductance

Net photosynthesis rate (P_N) and stomatal conductance (g_S) were measured under a light regime of 2000 μmol m^{-2} s^{-1} (saturation point) in peanut plant using LI-6400 portable photosynthesis system (LI-COR, Co., Ltd., Lincoln, Nebraska, USA). The second fully expanded leaf from the top was selected for P_N measurement. P_N in intact leaf was measured in the normal way as describe in Materials and Methods 2.3.3 in Chapter 2. In another way, P_N was measured in excised leaves. First, P_N in an intact leaf attaching to the plant was measured under 2000 μmol m^{-2} s^{-1} for about 10 min until the reading was stable. Then the leaf was excised off from the plant and P_N was measured under the same light continuously for about 40 min. The data of P_N and g_S (for vapor) were automatically recorded every 1 min. The value of g_S to CO_2 into a leaf was calculated as stomatal conductance to vapor multiplied by a coefficient of 0.62 because of the lighter molecular mass and faster diffusion of H_2O relative to those of CO_2.

8.3.3 Mathematical analyses for oscillations of photosynthesis and stomatal conductance

The oscillations or the cyclic changes in P_N or g_S in the intact leaf of peanut plants were analyzed by $P_N=[P_A+P_A\cos(\omega t+\tau)](1+\alpha t)+P_R(1+\beta t)$, or $g_S=[g_A+g_A\cos(\omega t+\tau)](1+\alpha t)+g_R(1+\beta t)$, where P_A or g_A is amplitude of oscillation of photosynthesis (P_N) or stomatal conductance (g_S); P_R or g_R is residual values of P_N or g_S at the first oscillation bottom; t is the time; ω is angular frequency; τ is the time needed for P_N or g_S to move into the oscillation process; α is the constant related to the decay of the oscillation cycles; β is the constant related to the dynamic change of P_R or g_R. In the equation, ω is defined as $\omega=2\pi f=2\pi/T$, where f is oscillation frequency; T is the period of oscillation. Thus, $T=\omega/2\pi$. In the original cosine trigonometric function, the oscillation takes the abscissa as central axis with negative values under the abscissa. In the photosynthetic or stomatal oscillation function, there was no negative value and

therefore the whole function was moved a scale up with the value of P_A added in the equation. Since the oscillation amplitude changes as time escapes, a factor of $(1+\alpha t)$ is multiplied. If the value of α is negative, the oscillation amplitude is decompressed as time escapes and If α is positive, the amplitude is amplified as time escapes. Even when P_N reaches the bottom during the oscillation, it still shows a positive value because the photosynthesis still continues through cuticular pores and the stomata that are not completely closed. Therefore, the residual value of photosynthesis, P_R, is added to the equation. Moreover, as P_A does, P_R also drifts up or down and therefore a factor of $(1+\beta t)$ is multiplied. Positive and negative values show the P_R drifting up and down, respectively. In the equation, the phase constant τ is actually the time needed for P_N to move into the oscillation process. Usually, photosynthesis cannot move into oscillation process immediately when the plant is place under light and therefore a time is needed.

For an excided leaf under a given light intensity, the photosynthetic rate or stomatal conductance would decline in a minus exponential manner as the leaf lost its water by transpiration. However, in some cases, especially in peanut plants, stomatal oscillation occurs in response to the leaf water loss. The oscillations are embedded in the minus exponential declines. There was a first exponential decrease in P_N or g_S once the leaves were excised and then an oscillation occurred accompanying the decline in P_N or g_S. Therefore, the oscillations of P_N or g_S in the excised leaves of peanut plants were modified as $P_N=[\exp(-\alpha_P t)]\{P_A+P_A\cos[\omega_P(t-\tau_P)]\}+P_R(1-\beta_P t)$, or $g_S=[\exp(-\alpha_S t)]\{g_A+g_A\cos[\omega_S(t-\tau_S)]\}+g_R(1-\beta_S t)$. The meaning of some coefficients was changed, i.e., P_A or g_A is the amplitude of the first oscillation after P_N or g_S starts decaying; α is the constant related to the decay of the exponential and oscillation cycles.

8.3.4 Analysis of water retention ability of the excised leaf

The transpiration declining curve for the excised leaves was analyzed according the same methods as described in Materials and Methods 3.3.11 in Chapter 3.

8.4 Results

8.4.1 Modified AnM increased oscillations of stomatal conductance and leaf photosynthesis in the intact leaves

Stomatal conductance (g_S) oscillations and the consequent net photosynthetic rate (P_N) oscillations are shown in Fig. 8.1. The oscillation curves of g_S and P_N were clearly different between peanut plants in modified AnM and control. The oscillation amplitudes P_A and g_A of photosynthesis (P_N) and stomatal conductance (g_S) were higher in modified AnM than in control, which was also attributed to higher maximum values and lower base values of P_N and g_S (P_A and g_A) (Table 8.1). This suggested that stomata in leaves of peanut plants in modified AnM were sensitive to environmental stimuli and could fully close or open compared with those in control. The period for one oscillation cycle ($T=\omega/2\pi$) was shorter in peanut plants in modified AnM than in control. This might be also attributed to the sensitivity of AnM plants to environmental stimuli. There was no difference in τ, the time needed to go into the oscillation, between treatments. Values of α for both P_N and g_S were lower in peanut plants in modified AnM than in control. This meant that the decays of all the capacity rates, the oscillation amplitudes and the base rates of P_N and g_S were smaller in modified AnM than in control.

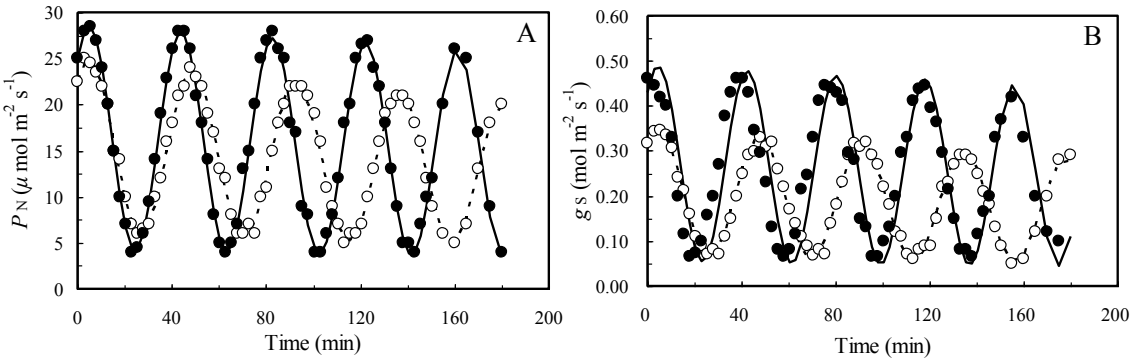

Fig. 8.1 Oscillations of photosynthetic rate (A) and stomatal conductance (B) in the intact leaves of peanut plants grown with the modified AnM technique (-●-) in comparison with control (-○-).

Table 8.1 Parameters from the analysis of the oscillation curves of stomatal conductance and photosynthetic rate in the intact leaves of peanut plants grown with the modified AnM technique.

Treatment	----------Stomatal conductance----------						
	g_A	g_R	ω	T	τ	α	β
	(mol m^{-2} s^{-1})		(min^{-1})	----(min)-----		-------(min^{-1})--------	
Control	0.141	0.067	0.145	43.3	4.7	-0.000963	-0.001476
Modified AnM	0.217**	0.054**	0.187**	37.8**	4.6^{ns}	-0.000493**	-0.001069**
	----------Photosynthetic rate----------						
	P_A	P_R	ω	T	τ	α	β
	(μmol m^{-2} s^{-1})		(min^{-1})	----(min)-----		-------(min^{-1})--------	
Control	9.3	6.2	0.143	43.9	4.8	-0.000983	-0.001485
Modified AnM	12.1**	4.3**	0.161*	39.1*	4.7^{ns}	-0.000485**	-0.001085**

P_A or g_A, amplitude of oscillation of photosynthesis (P_N) or stomatal conductance (g_S); P_R or g_R, residual values of P_N or g_S at the first oscillation bottom; ω, angular frequency; τ, the time needed for P_N or g_S to move into the oscillation process; α, the constant related to the decay of the oscillation cycles; β, the constant related to the dynamic change of P_R or g_R; T, the period of oscillation. *, ** and ns, significant differences at $P \leq 0.05$ and $P \leq 0.01$ and no significant difference, respectively.

8.4.2 Oscillations of stomatal conductance and photosynthesis in the excised leaves were clearly found in modified AnM

The oscillation curve for the excised leaf showed embedding of two phases of decreasing in P_N or g_S, the exponential declining phase and the oscillating phase (Fig. 8.2). Photosynthetic oscillation was synchronized to the movement of stomata. In most studies, the oscillation was found in leaves alive attached to the intact plant under given conditions and light intensity. Here, leaves were excised from the plant once photosynthesis got a maximum at 2000 μmol m^{-2} s^{-1} *PPF*. It is speculated that the leaf close its stomata in response to the sudden stop of water supply, consequently causing the sharp drop of stomatal conductance (g_S) and leaf photosynthetic rate (P_N). Therefore, exponential factor exp(-αt) instead of (1+αt) was multiplied in the function equation. After closing enough, stomata turned to a tendency of recovery to open from closure, leading to a cyclic variation of g_S during the period of closure. The oscillations of g_S or P_N were found in the excised leaves of plants in modified AnM but were not clear in control. The parameters analyzed from the modeling equations are presented in Table 8.2. The oscillation parameter such as the oscillation amplitude of P_N or g_S (P_A or g_A), the residual value of P_N or g_S (P_A or g_A), the angular frequency (ω), and the coefficient β, all existed in both curves for modified AnM and control. However, clear oscillation cycle only appeared once in the curve for modified AnM and was not clear in the curve for control. The oscillation tendencies were sheltered by the minus exponential declining trends. In other words, the oscillation capacities were diminished by cutting the leaves off from the plants. The stomata conductance would oscillate if the leaf was not cut off. Since the exponential tendency much diminished the oscillation, the factor (1-αt) for the intact leaves was omitted for the excised leaves. The comparisons or difference between modified AnM and control in the oscillation parameters were similar to those for the intact leaves. The oscillation amplitude of P_N or g_S (P_A or g_A) was higher but the residual value of P_N or g_S (P_R or g_R) was lower, and the cycle period (T_P) was shorter in leaves in modified AnM than in control. The clear difference in the exponential coefficient α was much higher for peanut leaves in control than in modified AnM. This explained why oscillation cycles were not clear in leaves in control but existed in leaves in modified AnM. It was suggested that the capacity of the oscillation tendency was stronger or the physiological activities in response to environmental changes were higher in leaves in modified AnM than in control.

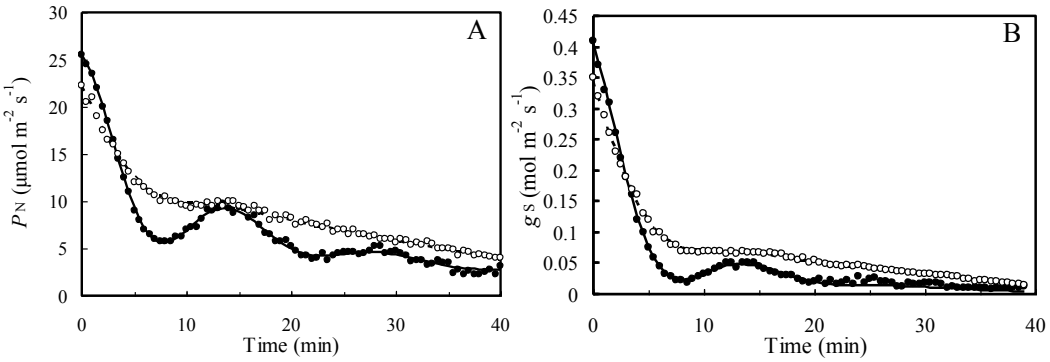

Fig. 8.2 Oscillations of leaf photosynthesis (A) and stomatal conductance (B) in the excised leaves of peanut plants grown with the modified AnM technique (-●-) in comparison with those in control (-○-).

Table 8.2 Parameters from the analysis of the oscillation curves of stomatal conductance (g_S) and photosynthetic rate (P_N) in the excised leaves of peanut plants.

Treatment	----------Stomatal conductance----------						
	g_A	g_R	ω_S	T	τ	α_S	β_S
	(mol m^{-2} s^{-1})		(min^{-1})	----(min)-----		-------(min^{-1})--------	
Control	0.114	0.089	0.347	18.1	0.49	0.245	-0.0217
Modified AnM	0.177**	0.028**	0.417**	15.1**	0.39^{ns}	0.179**	-0.0224^{ns}
	----------Photosynthetic rate----------						
	P_A	P_R	ω_P	T	τ	α_P	β_P
	(μmol m^{-2} s^{-1})		(min^{-1})	----(min)-----		-------(min^{-1})--------	
Control	4.7	11.8	0.355	17.7	1.24	0.219	-0.016
Modified AnM	8.6**	6.2**	0.452**	13.9*	0.98**	0.106**	-0.015^{ns}

P_A *or* g_A, the amplitude of the first oscillation after P_N or g_S starts decaying; P_R *or* g_R, residual values of P_N or g_S at the first oscillation bottom; α, the constant related to the decay of the exponential and oscillation cycles; ω, angular frequency; τ, the time needed for P_N or g_S to move into the oscillation process; β, the constant related to the dynamic change of P_R or g_R; T, the period of oscillation. *, ** and ns, significant differences at $P \leq 0.05$ and $P \leq 0.01$ and no significant difference, respectively.

8.4.3 Better leaf water retention ability in modified AnM contributed to the larger oscillation of leaf photosynthesis and stomata conductance

In response to the sudden stop of water supply, the leaves in modified AnM lost water faster at the beginning by higher stomatal conductance (shown by value of α) in comparison with leaves in control (Table 8.3). The fast loss of water caused the lower leaf relative water content at stomata closure (ζ_{SC}) in leaves in modified AnM with no difference in the time (t_{SC}) at which stomata closed. After stomata closed, the water loss tended to slow through cuticular transpiration. The value of β, which was proportional to cuticular transpiration, was smaller for leaves in modified AnM than control. The constant τ_D was the time when the leaf is air dried remaining 10% of relative leaf water content. AnM prolonged the time for leaf drying. The better leaf water retention ability in modified AnM would be considered to contribute to the larger oscillation of leaf photosynthesis and stomata conductance.

Table 8.3 Variables from the analysis of excised leaf transpiration declining curves.

Treatment	ζsc	t_{SC}(min)	α	β	τ_D (min)
Control	0.789	33.7	0.0762	0.00831	1197
Modified AnM	0.756^{*}	34.2^{ns}	0.0925^{**}	0.00652^{**}	1513^{**}

The curve was modeled as $\zeta=[\zeta_{sat}-\zeta_{SC}(1-\beta t)]e^{-\alpha t}+\zeta_{SC}(1-\beta t)$. ζ_{SC}, the leaf relative water content at the time when the stomata closed; β, a constant related to cuticular transpiration; α, a constant related to the stomatal transpiration; t_{SC}, time when stomata started to close; τ_D, the time used to dry the excised leaf to a relative water content of 0.10. *, ** mean significance at $P\leq0.05$, at $P\leq0.01$ according to LSD analysis.

8.5 Discussion

In my previous experiments, it was found that physiological activities, plant growth and the final shell yield of peanut crops was improved by exposing the hypocotyls in the AnM techniques and these improvements were attributed to xerophytophysiological regulations such as osmotic adjustment and leaf surface morphological strengthening. In the present experiment, it was found that stomata in leaves of peanut plants gown in modified AnM were more sensitive to environment changes, closing completely when adverse conditions were perceived and trying to open again when leaf water was in relative balance. This was confirmed by the different types of stomatal oscillations in intact leaves and in excised leaves of peanut plants in modified AnM compared with those in control. It was suggested that peanut leaves in modified AnM could maintain higher stomatal conductance at the beginning and recovered to try opening stomata again but could retain the leaf water by completely closing stomata and by the low cuticular conductance at the later stages of the leaf drying period. This might be related with the physiological sensitivities and dehydration resistance in peanut leaves that was enhanced by exposing the hypocotyl in the AnM technique, as described in the previous chapters. The physiological significance of the damped or decaying photosynthetic or stomatal oscillation will be further studied.

Actually, stomatal movement is controlled by many complicated processes. In general, stomata open during the day and close in night following the circadian rhythm (Tallman 2004), which is different from environment responsive oscillation. Stomatal oscillation is usually found after a sudden change in one environmental factor in relatively constant environments (Cowan 1972ab). Many environmental stimuli, e.g., low humidity, strong light, drought, can induce stomatal oscillation (Hirose et al. 1992). These stimuli interact with unstable leaf water uptake on stomatal opening to adjust the balance between root water uptake and transpiration water loss even under sufficient soil water conditions (Van der Veen 1949; Barrs 1971; Hirose et al. 1991; Xu et al. 1994). The mechanisms as to how stomatal oscillations are initialized and regulated can be summarized at physiological, cellular and molecular levels. At the physiological level, the concept that oscillations result from instability in the negative feedback loops that control stomatal aperture via leaf water status seems to be widely accepted (Barrs 1971; Hirose et al. 1991; Yang et al. 2005; Marenco et al. 2006; Stepper et al. 2006). As found in the intact leaves attaching to the peanut plants in present experiment, stomata closed in response to the high evapotranspiration demand shown by high light intensity, high temperature and low air humidity, whereby water uptake from the root to leaves could not balance the water loss by transpiration. When stomata closed, the leaf was recharged by the steady water uptake from the root to the leaf and the stomata opened again, forming stomatal conductance oscillations and the consequent oscillation of photosynthetic rate. This was the so-called negative feedback loop of stomatal oscillation. In addition, positive feedback control may also be involved in the regulation

of stomatal apertures and this hydraulic effect may increase the tendency for stomatal oscillation (Kaiser and Kappen 2001; Yang et al. 2005; Stepper et al. 2006). At the cellular level, stomatal oscillations may be induced in two ways: a quicker responsive way with the variations in guard cell membrane tension and a slower way with the change in guard cell osmotic potential mediated by complex signaling, such as ABA and Ca^{2+} signaling (Tallman 2004; Pei and Kuchitsu 2005; Yang et al. 2005; Qiu and Zhang 2010), which requires further investigation. Water channels (a large family of proteins called aquaporins) are also involved in stomatal oscillation (Yang et al. 2006). Environment stimuli may initialize the signal cascade, Ca^{2+} or other signals such as ABA, H^+, Cl^-, and K^+, to regulate water channel by (de)phosphorylation (Yang et al. 2005). The mechanisms of water channel regulation await further clarification.

Stomatal oscillation is a widespread occurrence under relatively stable conditions, but this does not mean that stomatal oscillation could happen when the conditions are completely kept stable. In fact, stomatal oscillation generally occurs when some of the environmental factors change suddenly even if the change will not impose a stress on plant. Plants lose large amount of water daily through stomatal transpiration. This should not be a problem if adequate water is available, but under stressful conditions, simple stomatal opening and closing may not serve to control this balance. However, stomatal oscillation may be beneficial in optimizing the relationship between CO_2 absorption and water loss, improving water use efficiency under limited water conditions and plant resistance to environmental stress. A resistance-inductance-capacitance circuit model shows that stomatal oscillation is regulated by the transpiration pulling force and water transport resistance (Yang et al. 2005). How to utilize and regulate stomatal oscillation is an interesting challenge that needs further exploration. Nevertheless, hardening plants as done in the present experiment by exposing hypocotyls of peanut seedlings might increase the physiological sensitivities and activate the mechanisms of stomatal oscillation to avoid damages by environment changes.

Chapter 9

Enhancement of Activities of Antioxidant Enzymes in Response to Hypocotyl Exposure in Peanut Seedlings

9.1 Abstract

In the previous experiments, the AnM techniques (exposing the extra-elongated hypocotyls and then earthing the soil up to welcome late pegs) proved effective in improvements of plant growth and pod yield by inducing xerophytophysiological regulations such as osmotic adjustment in peanut crops. The key step of AnM techniques is the hypocotyl exposure treatment. In the present experiment, production of superoxide radicals and malondialdehyde (MDA) and the activation of the antioxidant enzymes were examined to elucidate the possible relations of the antioxidant systems to the growth improvement. Superoxide radical was increased but no production of MDA was observed during early period of the hypocotyl exposure treatment, which suggested that there was no damage to plants by the exposure stimuli. Moreover, the super superoxide dismutase (SOD) was activated but peroxidase (POD) and catalase (CAT) were not and this also suggested that hypocotyl exposure was not a real stress but a mild stimulation. Nevertheless, the hypocotyl exposure sufficiently induced xerophytophysiological regulations such as osmotic adjustment, which ensured the improvements in plant growth, physiological activities and pod yield.

Key words: AnM, antioxidant enzymes, hypocotyl, peanut (*Arachis hypogaea* L.), superoxide radicals, super superoxide dismutase (SOD), xerophytophysiology.

9.2 Introduction

In the previous experiments, the AnM techniques have proved effective in improvements of plant growth and pod yield in peanut crops. The technique has been modified in combination with film mulch and alternated by seedling transplanting. Both the modified and alternated AnM techniques showed yield improving effects similar or superior to the basic AnM technique in peanut crops. The main point of all the AnM techniques is the treatment of exposing the hypocotyl to light and dry air to induce xerophytophysiological regulations. Examinations have confirmed that hypocotyl exposure results in osmotic adjustment and the consequent improvement in leaf turgor potential, which is the driving force of cell enlargement in plant growth and stomatal opening in photosynthetic processes. Exposing the hypocotyl to light and dry air imposes drought stimulation to the peanut plant although there is no real water deficit in the soil. The stimulation might cause similar responses from the plant as the real drought stress can. The existence or absence of the stress can be tested by examining the oxidants and antioxidant enzyme systems in cells of the plants (Xu et al. 2011d). When subjected to environmental stress such as drought (Sharma and Dubey 2005), oxidation in cells is enhanced to produce more free radicals or reactive oxygen species (ROS). ROSs are formed in biological systems as part of normal metabolism but the level of ROS gets higher than normal if the plant is under environmental stresses, such as drought, high irradiation including UV, extreme temperatures, pesticides, ozone, plant metabolic activity, and nutrient deficiencies (Bischof et al. 2003). Although ROSs are detrimental to plants, ROS such as H_2O_2 acts as a signaling molecule and second messenger, which may positively arouse the defense systems to avoid damages (Mulligan et al. 1997; Scheel and Wasternack 2002; Bhattacharjee 2005; Walley and Dehesh 2010). To avoid damages caused by these excess ROS, plants have developed a series of antioxidant enzyme systems with elaborate mechanisms. In higher plants, an antioxidant system, which prevents the formation of ROS consist of 1) low molecular mass antioxidants that work as ROS scavenger such as ascorbic acid, glutathione, tocopherols, some phenolic compounds such as flavonoids, tannins and lignin precursors, 2) enzymes that regenerate the reduced forms of antioxidants, and 3) enzymes that reduce the formation of ROS (Blokhina et al. 2003). Plant cells are protected against oxidative stress by an interacting network of these antioxidant enzymes. The main antioxidant enzymes include superoxide dismutase (SOD), catalase (ACT) and peroxidase (POD) (Chelikani et al. 2004; Raychaudhuri and Deng 2008). When acclimating to increased levels of oxidative stress caused by adverse environmental conditions such as drought (Contreras-Porcia et al. 2011), extreme temperatures and excess and strong irradiation, SOD concentrations typically increase with the degree of stresses (Alscher et al. 2002). When subjected to water stress, the drought resistant varieties always show higher transcript level of antioxidant enzymes than the susceptible ones (Smirnoff 1993). Malondialdehyde (MDA) is a marker for

oxidative stress (Janero 1990; Del Rio et al. 2005). Polyunsaturated lipids are degraded by reactive oxygen species and form MDA (Pryor and Stanley 1975). MDA is a reactive aldehyde, which causes toxic stress in cells (Farmer and Davoine 2007). MDA reacts with deoxyadenosine and deoxyguanosine in DNA, forming mutagenic DNA adducts (Marnett 1999). Quantification of primary lipid peroxidation products is difficult because reactive nature of these compounds is not stable (Gray 1978; Janero 1990). Thus, analysis of the secondary oxidation products such as MDA is adopted to quantify lipid peroxidation. In some cases, activities of SOD, CAT and POD keep higher in drought resistant varieties than the susceptible one after the water stress is released, suggesting that CAT, POD, and APX are also associated with better post-drought recovery from drought stress (Xu et al. 2011g). The activities of total SOD and ascorbate peroxidase show consistent increases with increasing extent of drought stress. Some PODs such as guaiacol POD and chloroplastic ascorbate POD show high activity under mild drought stress but the activity declines under severe drought stress (Buchholz et al. 1995). The activity of diverse antioxidant enzymes increased during desiccation and the high activity diminished to near basal levels during rehydration. However, if the stress is too strong, the defense system can not remove the high production of ROS effectively and result in damage to plants (Buchholz et al. 1995).

In the present research, the treatment of exposing the hypocotyls of peanut seedlings with the root anchored in moist soils is just a mild stimulation but not a real drought stress. The stimulation is expected to induce xerophytophysiological regulations including signal transduction, gene activition and transcription and the appropriate physiological adjustments. Oxidation and production of ROS and the consequent activation of the antioxidant enzymes might be involved in the processes of xerophytophysiological regulations. Therefore, in the present experiment, production of ROS and MDA, the activation of the antioxidant enzymes such as SOD, POD and CAT are examined to confirm the stimulation of the treatment of exposing hypocotyls in the AnM techniques in peanut cultivation.

9.3 Materials and methods

9.3.1 Plant materials and treatments

Seeds of a Japanese peanut cultivar (*Arachis hypogaea* L. cv. Chibahandachi) are sown in planters (50 × 40 × 20 cm) filled with a fine Andosol (50%) mixed with peat moss (50%). The planters are placed outdoors under natural conditions from middle May to October. The seeds are sown 8 cm deep in planter to induce the extra-elongation of the hypocotyls. This experiment was to simulate the "A" and "n" stage in the AnM practice in field conditions. Soils around 7 day old seedlings were removed with the hypocotyls exposed, which were subjected to light and dry air (Fig. 9.1).

Fig. 9.1 Treatments of inducing the extra-elongation and exposing the hypocotyl of peanut seedlings (left, control; right, hypocotyl exposure treatment).

9.3.2 Measurements of soluble proteins and antioxidant enzymes

Total soluble proteins (or the activated enzyme solution) were extracted by pH 7.5 50 mM phosphate buffer solution and determined according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford 1976; Murphey et al. 1989; Elavarthi and Martin 2010). Standards were prepared as a range of 0 to 150 µg protein (BSA). Its absorbance at 595 nm was determined after 2 min and before 1 hour using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The content of soluble proteins was expressed as µg per g of fresh weight.

The free superoxide radical ($O_2 \cdot^-$) generation was measured as described by Bissenbaev et al. (2007) by monitoring the nitrite formation from hydroxylamine in the presence of $O_2 \cdot^-$, with some modifications. The incubation mixture contained 0.5 ml enzyme extract, 0.5 ml 50 mM phosphate buffer (pH 7.5), 1 ml 1 mM hydroxylamine hydrochloride. After incubation at 25˚C for 1 h, the 1 ml 17 mM sulfanilamide and 1 ml 7 mM α-naphthylamine were added to the incubation mixture. The reaction mixture was incubated at 25˚C for 20 min. The absorbance was measured at 530 nm using a spectrometer (Hitachi U-2000, Tokyo, Japan). A standard curve with NO_2^- was used to calculate the production rate of $O_2 \cdot^-$.

The superoxide dismutase (SOD) activity was determined using riboflavin-nitroblue tetrazolium (NBT) assay according to Beyer and Fridovich (1987) and Chakrabarty et al. (2009). The enzyme extract (100 µl) was first mixed with 2.9 ml of 50 mM phosphate buffer (pH 7.5) containing 10 µM EDTA, 13 mM Met, and 75 µM NBT, 2.0

μM riboflavin then added. The reaction mixture was illuminated under 135 μmol m^{-2} s^{-1} light for 5 min and the absorbance was read at 560 nm. One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the riboflavin-mediated reduction of NBT.

Catalase (CAT) activity was measured by deletion of H_2O_2 at 240 nm (Beers and Sizer 1952). One unit of CAT gives an H_2O_2 decomposition rate of 1 μmol min^{-1} at 25˚C.

Peroxidase (POD) activity was assayed by oxidation of guaiacol as substrate (Khalil et al. 2006). Typical reaction mixture contained 100 μl of enzyme extract, 2.9 ml of 50 mM phosphate buffer (pH 5.5), 1 ml of 50 mM guaiacol, and 1 ml of 2% H_2O_2. The product formation was followed spectrophotometrically at 470 nm. One unit of POD activity was defined as the quantity of the enzyme that oxidizes 1 μM guaiacol per min at 25˚C.

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipid, using the thiobarbituric acid method (Heath and Packer 1968; Dey et al. 2007). The enzyme extract 1 ml was mixed with 2 ml TBA reagent (0.6%). The reaction mixture was heated at 100˚C for 30 min and terminated in an ice bath and centrifuged at 4500 rpm for 10 min. the absorbance of the colored supernatant was measured at 532 nm and corrected for non-specific absorbance at 450 nm and at 600 nm. The concentration of MDA (μmol l^{-1}) was calculated as 6.45×(OD532-OD600)-0.56 ×OD450.

All assays were conducted at 25˚C, and enzyme activity was expressed as Units per mg protein.

9.3.3 Analysis of the dynamic accumulation of plant dry mass

Plant samples were taken after treatment at internals of 1 to 4 days during the hypocotyl exposure treatment period and at internals of around 10 days after treatment until harvest. Graphs were separately plotted for the treatment period and for the whole growth period. The detailed descriptions for the dynamics of changes in dry mass production during the whole growth period were reported in Materials and Methods 3.3.6 in Chapter 3.

9.3.4 Analysis of leaf photosynthesis

Analysis of leaf photosynthesis was made as described in Materials and Methods 2.3.3 in Chapter 2. The leaf color was measured using a chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Konica Minolta Sensing, Inc., Tokyo, Japan) after the photosynthetic measurement.

9.3.5 Analysis of osmotic adjustment by the P-V curve method

Osmotic adjustment was made just after the hypocotyl exposure and evaluated by analyzing the pressure-volume (P-V) curve with the same method as in Materials and Methods 2.3.4 in Chapter 2.

9.4 Results

9.4.1 Hypocotyl exposure increased production of superoxide radicals

As shown in Fig. 9.2 A, concentration of free superoxide radical ($O_2 \cdot^-$) in hypocotyl was significantly higher than in control after the hypocotyl was exposed until 9 days after the exposing treatment. The $O_2 \cdot^-$ producing rate in hypocotyl shown as nmol per mg of protein per min was also significantly higher in exposed seedlings than in control seedlings although the trends showed steady decreasing in both exposed and control seedlings (Fig. 9.2 B). Dynamic changes in concentration of $O_2 \cdot^-$ in leaf were different from those in hypocotyls with a trend of first decreasing and then increasing again (Fig. 9.3 A). At most of the measurement days, $O_2 \cdot^-$ in leaf was higher in hypocotyl-exposed seedlings than in control seedlings. The dynamic changes in producing rate of $O_2 \cdot^-$ in leaf showed a similar trend to that in $O_2 \cdot^-$ concentration (Fig. 9.3 B). The results suggested that oxidative stress, no matter severe or not, did occur in hypocotyl and leaf of the hypocotyl-exposed peanut seedlings in comparison with the control seedlings.

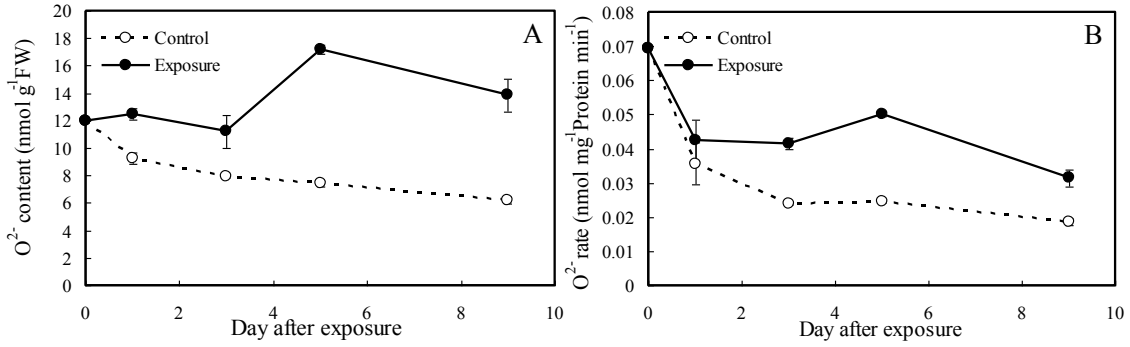

Fig. 9.2 Dynamical changes in reaction of superoxide radical content (A) and in production rate of superoxide radical (B) in the hypocotyls of peanut seedlings. Error bars represent SE values for three independent replicates.

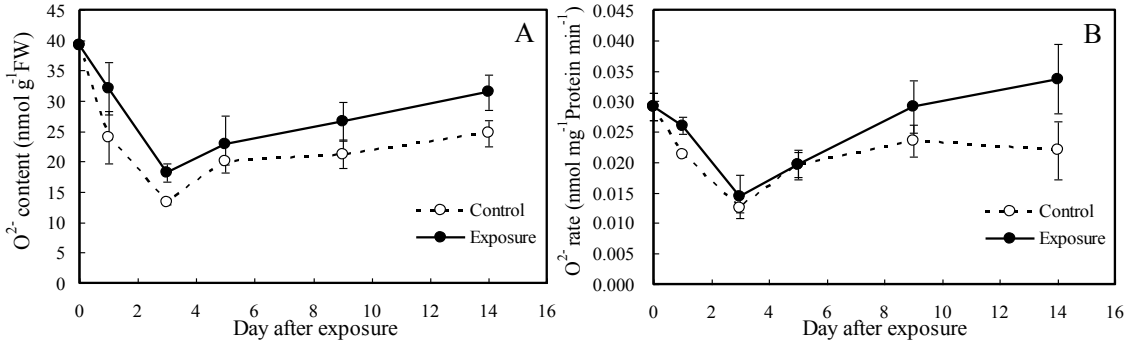

Fig. 9.3 Dynamical changes in reaction superoxide radical content (A) and in production rate of superoxide radical (B) in leaves of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.2 Hypocotyl exposure did not enhance MDA production

The damage induced by oxidative stress can be demonstrated by measuring malondialdehyde (MDA) production, representing a product of reactive oxygen species (ROS) induced lipid peroxidation. The concentration of MDA in hypocotyl was not higher, sometimes lower in exposed seedlings than in control seedlings with the exception at the 9th day, when it was higher in exposed than control seedlings (Fig. 9.4 A). The MDA concentration in leaf was also not higher, sometimes lower in exposed than in control seedlings with the exception at the 9th day (Fig. 9.4 B). However, it tended close up to the same at the 14th day. In overall, the results of dynamic changes in MDA concentration did not suggest enhancement of MDA production in hypocotyl-exposed peanut seedlings. In other words, there was no damage caused by hypocotyl exposure stimulation.

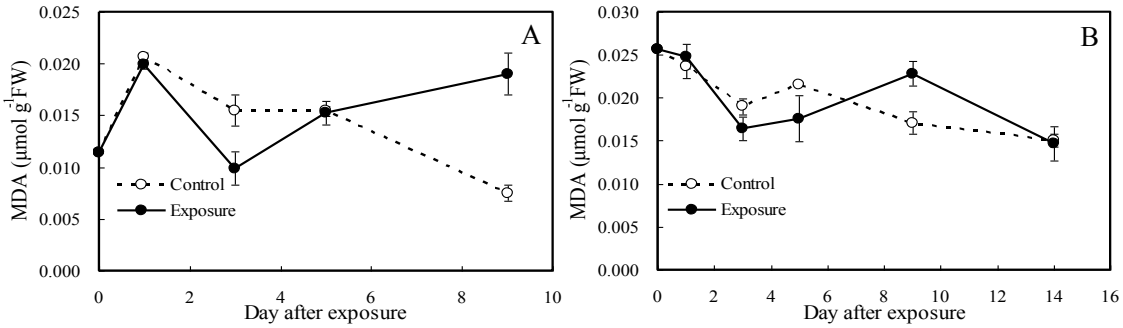

Fig. 9.4 Dynamic changes in MDA in hypocotyl (A) and leaf (B) of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.3 Hypocotyl exposure stimulation did induce increase in soluble proteins

Stress-induced protein degradation is a general phenomenon that affects many soluble proteins. In plants adapted to the stresses, protein synthesis increases and the initially rapid rate of proteolysis is reduced (Cooke et al. 1979). In the present study, with the exception at the third day, the content of soluble proteins in hypocotyl was significantly higher in hypocotyl-exposed seedlings than in control seedlings until 9 days after the exposing treatment (Fig. 9.5 A). The soluble proteins in leaf were higher in hypocotyl-exposed seedlings than in control seedlings with the exception at the 14th day (Fig. 9.5 B). The results suggested that hypocotyl exposure stimulation did induce increases in enzyme amount, which was shown by the soluble protein amount.

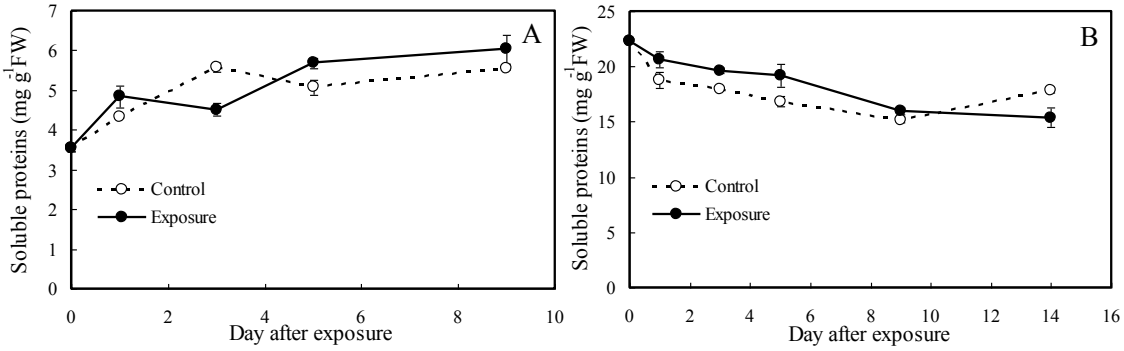

Fig. 9.5 Dynamical changes in soluble proteins in hypocotyl (A) and leaf (B) of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.4 Hypocotyl exposing stimulation induced activation of superoxide dismutase (SOD)

SOD activity in hypocotyl shown by both per g of fresh mass and per g of protein increased steadily and was significantly higher in hypocotyl-exposed seedlings than in control seedlings (Fig. 9.6 A, B). SOD activity in leaf expressed by both per g of fresh mass and per g of proteins showed a similar dynamic changing trend to each other, first increasing, then decreasing and increasing again. With the exception at the 9th day, SOD activity in leaf was significantly higher in hypocotyl-exposed seedlings than in control seedlings during the two weeks of hypocotyl exposure treatment (Fig. 9.7A, B). The abovementioned results suggested that hypocotyl exposing stimulation did induce activation of the antioxidant enzyme, superoxide dismutase (SOD), which was responsible to remove the ROS and to prevent cells from oxidative damages.

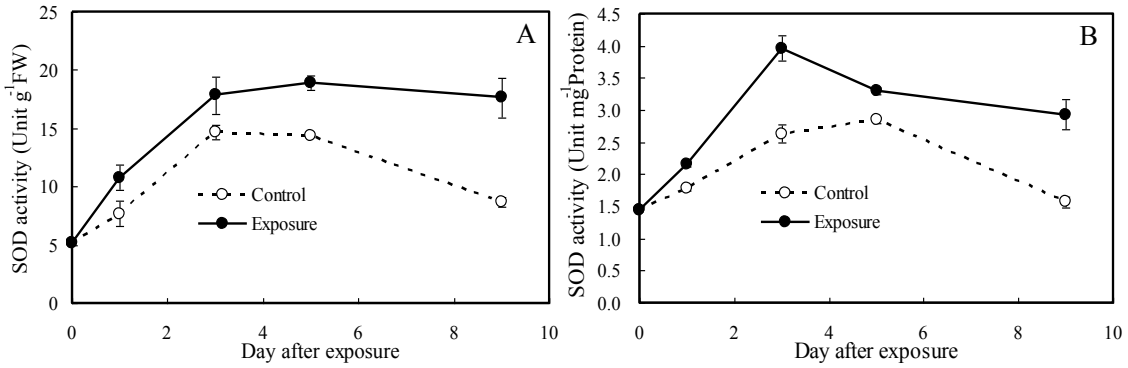

Fig. 9.6 Dynamical changes in SOD activity in hypocotyl of peanut seedlings. Error bars represent SE values for three independent replicates.

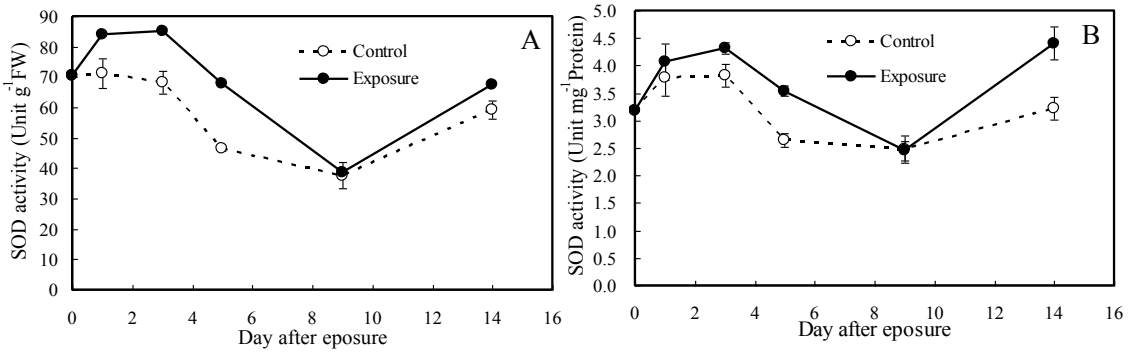

Fig. 9.7 Dynamical changes in SOD activity in leaf of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.5 POD was not sensitive to the early treatment of hypocotyl exposure

POD activity in hypocotyl increased steadily and was lower in hypocotyl-exposed seedlings than in control seedlings up to 3 days after the treatment and then got higher than in control seedlings (Fig. 9.8 A). The POD activity in leaf of hypocotyl-exposed seedlings showed large fluctuations by hypocotyl exposure but was also lower in the first three days and got higher than in control seedlings (Fig. 9.8 B). From the results, it was suggested that hypocotyl exposure stimulation did not induce activation of POD during the first three days and induced the enzyme activity during the later period of the treatment, which also implied that POD was not sensitive to the hypocotyl exposure, a slight stimulation.

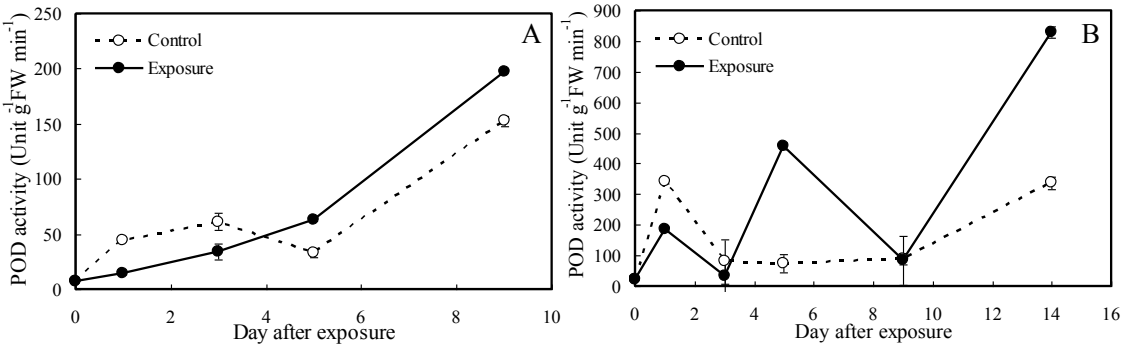

Fig. 9.8 Dynamical changes in POD activity in hypocotyl (A) and in leaf (B) of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.6 CAT was sensitive to hypocotyl exposing stimulation in hypocotyl but not in leaf

As shown in Fig. 9.9 A, CAT activity in hypocotyl was higher in hypocotyl-exposed seedlings during the first several days but got lower in the later period of hypocotyl exposure in comparison with the control seedlings. CAT activity in leaf showed the opposite changing trends to that in hypocotyl (Fig. 9.9 B). These results suggested that CAT was sensitive to exposing stimulation in hypocotyl, which directly received the stimulation, but not sensitive in leaf, which did not directly receive the stimulation.

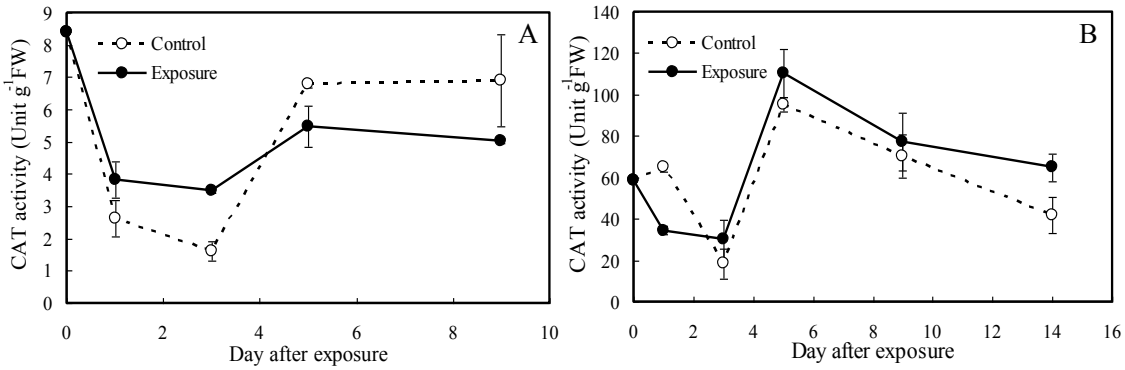

Fig. 9.9 Dynamical changes in CAT activity in hypocotyl (A) and leaf (B) of peanut seedlings. Error bars represent SE values for three independent replicates.

9.4.7 Long-term dry mass accumulation and final pod yield was improved by hypocotyl exposure

Soon after the hypocotyl exposure treatment was released from 25 days, dry mass per plant was steadily higher than in the control plant (Fig. 9.10 A). The dry mass accumulation of pods showed a dynamic trend similar to that of plant dry mass and was more clearly different between hypocotyl-exposed plants and the control plants (Fig. 9.10 B). The parameters analyzed from the sigmoid curve are shown in Table 9.1. The maximum dry mass (G_M) for both the whole plant and pods was higher in hypocotyl-exposed plant than control plant. Because the dry mass accumulation started from the seed, there was no difference in G_B. As shown by τ (day), peanut plants with hypocotyl exposed reached half of G_M 2 or 3 days earlier for the whole plant and pods. The constants α and β also showed differences between treatments.

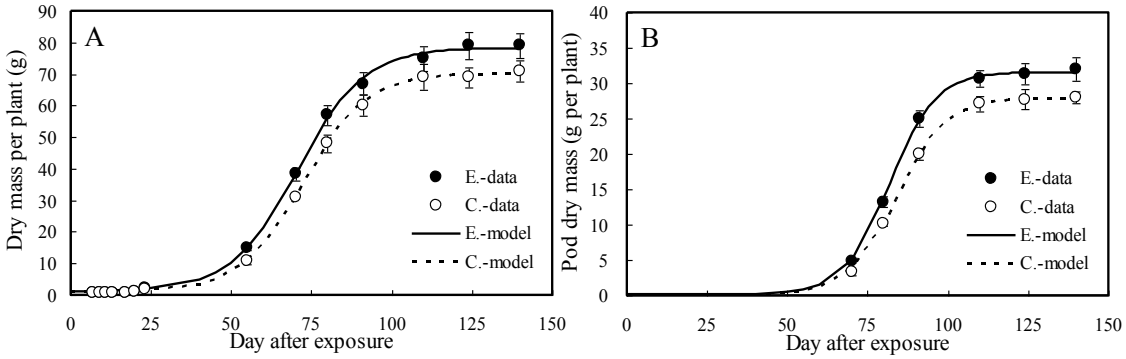

Fig. 9.10 Dry mass accumulation in the whole peanut plant (A) and in pods (B) with and without hypocotyl exposed. C., control; E., exposure; C.-data and E.-data, the original data; C.-model and E.-model, the model analyzed by $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$ according to the original data. Error bars represent SE values for three independent replicates.

Table 9.1 The growth analysis parameters for peanut plant and pod grown with and without hypocotyl exposure.

Treatment	----G_M (g pl^{-1})----		----G_B (g pl^{-1})----		----τ (day) ------		----------β--------		--------α--------	
	Plant	Pod	Plant	Pod	Plant	Pod	Plant	Pod	Plant	Pod
Exposure	77.5	31.5	1.1	0.1	94.5	83.5	0.00213	0.00232	0.094	0.138
Control	69.4	27.8	1.1	0.1	96.5	86.5	0.00219	0.00273	0.099	0.129
Statistic	**	**	ns	ns	*	*	ns	*	*	**

Parameters were analyzed by ANOVA. ns, no significance; *, significance at $P \leq 0.005$; **, significance at $P \leq 0.001$. The dynamics of changes in dry mass production during the whole growth period was modeled by $G = G_M[1+(1-\beta t)e^{-\alpha(t-\tau)}]^{-1}-G_B(1-\beta t)$. G_M was the maximum increment of biomass; G_B was the original biomass of the plant at the beginning; α was the constant related with the steep part of the curve; β was the constant related with sloping part of the curve before and after the fast growth; τ was the time point when the biomass increment reached half amount of G_M; and t was the growth time (day).

9.4.8 Hypocotyl exposure improved photosynthetic activity

At all photosynthetic photon flux (*PPF*) levels the net photosynthetic rate (P_N) was higher in leaves of hypocotyl-exposed peanut plants than in control plants (Fig. 9.11). The parameters analyzed from the photosynthetic response curves were shown in Table 9.2. Photosynthetic capacity (P_C), dark respiration rate (R_D) and the maximum quantum yield (Y_Q) as well as the chlorophyll content were higher in leaves of hypocotyl-exposed peanut plants than in control plants.

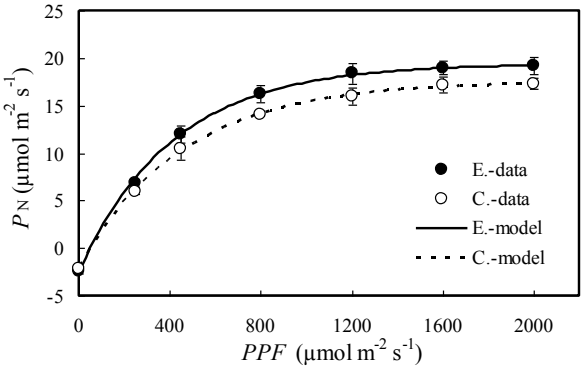

Fig. 9.11 Photosynthetic light response curves for peanut plants with and without hypocotyl exposure treatment. C., control; E., exposure; C.-data and E.-data, the original data; C.-model and E.-model, the model analyzed by $P_N = P_C (1\text{-}e^{-KI}) - R_D$. Error bars represent SE values for three independent replicates.

Table 9.2 Photosynthetic capacity (P_C), respiration rate (R_D), the maximum quantum yield (Y_Q) and chlorophyll level in peanut plant grown with and without hypocotyl exposure treatment.

Treatment	P_C	R_D	Y_Q	Chlorophyll
	----------(μmol m^{-2} s^{-1})----------		(mol mol^{-1})	(SPAD)
Exposure	21.9*	2.5*	0.0537*	55*
Control	19.7	2.1	0.0498	52

The photosynthetic light response curve was modeled by $P_N = P_C (1\text{-}e^{-KI}) - R_D$. Parameters were analyzed according to LSD analysis. *, significance at $P \leq 0.005$; **, significance at $P \leq 0.001$.

9.4.9 Hypocotyl exposure induced osmotic adjustment

As shown in Table 9.3, at fully saturated water status, the leaf water potential (Ψ_{FT}) was slightly lower but the osmotic potential (π_{FT}) was further lower and consequently the leaf turgor potential (P_{FT}) was significantly higher in hypocotyl-exposed peanut seedlings than in control seedlings. At midday when the transpiration demand was high and leaf water lost fast, the leaf turgor potential (P_{MD}) was higher because the osmotic potential (π_{MD}) was lower under the same leaf water potential (Ψ_{MD}). The improvement in leaf turgor potential was attributed to the active solutes accumulation shown by ΔC_{FT}, which showed the additional increase in solutes in comparison with the control plants. Osmotic potential in the symplastic solution theoretically diluted by apoplastic water (π_{s+a}) showed a similar trend to π_{FT}. The osmotic potential at incipient plasmolysis (π_{IP}) was lower in hypocotyl-exposed seedlings than in control seedlings, indicated that hypocotyl exposure improved water stress tolerance in peanut seedlings. The leaf relative water content at incipient plasmolysis (ζ_{IP}) showed no significant difference. The symplastic water fraction (ζ_{sym}) was larger and the apoplastic water fraction (ζ_{apo}) was smaller in hypocotyl-exposed seedlings than in control seedlings. It was suggested that hypocotyl exposing stimulation improved cell water conditions in the symplasm where biochemical functions were performed. The constants α and β were related with the shape of the P-V curve and both lower for hypocotyl-exposed seedlings than for control seedlings.

Table 9.3 The parameters from P-V curve analysis for peanut plants grown with and without hypocotyl exposure treatment.

Treatment	Ψ_{FT}	Ψ_{MD}	π_{FT}	π_{MD}	P_{FT}	P_{MD}	π_{s+a}	π_{IP}	C_{FT}	ΔC_{FT}	ζ_{IP}	ζ_{sym}	ζ_{apo}	α	β
	----------(MPa)----------								(osmol m^{-3})						
Exposure	-0.192	-0.82	-1.31	-1.37	1.12	0.55	-1.08	-1.57	537	115	0.821	0.847	0.153	55.8	0.963
Control	-0.208	-0.80	-1.03	-1.21	0.82	0.41	-0.81	-1.26	422	0	0.834	0.818	0.182	66.2	0.987
Statistic	*	ns	**	**	**	*	**	**	**	**	ns	*	*	*	*

Parameters were analyzed according to LSD analysis. ns, no significance; *, significance at $P \leq 0.005$; **, significance at $P \leq 0.001$.

The pressure-volume (P-V) curve was modeled as follows,

$$-\Psi^{-1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{apo}]$$

where, Ψ was leaf water potential at a level of relative leaf water content (ζ); Ψ_{FT} is Ψ at full turgor or in saturated leaf water conditions; π_{s+a} was the osmotic potential (π) in the symplastic solution theoretically diluted by apoplastic water; (1-ζ) was leaf water deficit as the independent variable in this equation; β was a coefficient used to adjust the slope of the less sloping part of the curve; α is a coefficient associated with the slope of steeply sloping part of the curve, and ζ_{apo} is apoplastic water fraction shown as $\zeta_{apo} = \zeta_{FT} - \zeta_{sym} = (1 - \zeta_{sym})$. ζ_{sym} was the symplastic water fraction and ζ_{FT} was ζ at full turgor with a value of 1.

9.5 Discussion

In the present research, peanut hypocotyl exposure in the AnM technique was used as a stimulation to induce positive xerophytophysiological regulations. When the hypocotyl, which usually remains in soil, is suddenly exposed to light and dry air, the radiation and dry air impose a drought-like stimulation to the peanut seedlings. Because the root system of the young peanut seedling anchored in the moist soil, there is no real drought or water stress to the plant. Even though there is a real stress, some very beneficial effects can be expected from the stimuli such as mild or partial drought, water deficit, radiations and low humidity (Turner 1990; Xu 2007).

In the present research, several experiments have proved that hypocotyl exposure, as one stimulus to the seedling, can be expected to induce positive regulations such as improvement in biomass production, pod yield, which are supported by leaf turgor maintenance from the osmotic adjustment ensured by active accumulations of osmolytes such as sugars, anthocyanins, amino acids and soluble proteins. Nevertheless, the sudden hypocotyl exposure to the light and dry air is a drought-like stimulation. The stimulation might induce the production of superoxide radicals, which are not only cause damages to the cell, if the level is high enough, but also act as signals to induce stress-tolerance regulations, or xerophytophysiological regulations in this case. Results of the present experiment confirmed that both the concentration of superoxide radical ($O_2\cdot^-$) and the $O_2\cdot^-$ producing rate in hypocotyl and leaf of hypocotyl-exposed peanut seedlings was significantly higher than in control. The results suggested that hypocotyl exposure as stimulation did induce increases in $O_2\cdot^-$ although it was just a mild or false drought stimulation. Although abscisic acid (ABA) is suggested as signal chemical caused by drought stimulation, superoxide radicals are also considered as signal chemicals, which might at least collaborate with ABA together to cause signaling (Jensen et al. 2008). The oxidation, no matter severe or not, did occur in hypocotyl and leaf by hypocotyl exposure.

Usually, the damage caused by $O_2\cdot^-$ can be confirmed by production of MDA, a product of ROS induced lipid peroxidation (Pan et al. 2006; Wang et al. 2011). The concentration of MDA in both hypocotyl and leaf was not higher, sometimes lower under hypocotyl exposure treatment (Fig. 9.4). It was suggested that MDA production did not really increase and there was no real or severe oxidative damages in hypocotyl exposed peanut seedlings although the superoxide radicals were really increased. This implied that hypocotyl exposure was just a mild stimulation instead a severe stress.

As signal chemicals, the increased superoxide radicals by hypocotyl exposure induced increases in soluble proteins in both hypocotyl and leaf, which showed the amount of enzymes (Fig. 9.5). Usually, mild stresses induce protein synthesis while severe stresses induce protein degradation and the same for activities of antioxidant enzymes. With maize (Li et al. 2007; Zhang et al. 2011), pigweed (Sun et al. 2011), soybean (Masoumi et al. 2011), *Betula maximowicziana* (Wang et al. 2011),

Rhododendron (Li et al. 2011) and *Glycyrrhiza uralensis* (Pan et al. 2006) as examples, activities of the antioxidant enzymes, mainly SOD, increased under mild drought stress but decreased under severe stress. According to Liu et al. (2011), when seedlings of *Cupressus funebris* were subjected to severe drought condition, activities of all SOD, POD and CAT increased consistently, but under mild drought or recovered water conditions, the SOD activity remained at a higher level than control but the POD and CAT activities showed the same level as control. They concluded that *C. funebris* seedlings could resist mild drought stress via increasing their soluble protein concentration and inhibiting membrane lipid peroxidation by activating SOD, but could not resist severe drought stress because of the irreversible damage of cell membrane structure. In plants adapted to stresses or just stimulated by mild stresses, protein synthesis increases and the proteolysis is reduced (Cooke et al. 1979). In the present experiment, when hypocotyl was exposed for two weeks, increases in activity of SOD but decreases in activity of POD and CAT were observed, suggesting the stress caused by hypocotyl exposure was deepening as the treatment was prolonged. Not only the amount of the enzymes but also the SOD activity in hypocotyl and leaf of hypocotyl-exposed peanut seedlings increased in response to stimulation treatment (Fig. 9.6-7). It was suggested that hypocotyl exposure did induce activation of the main antioxidant enzyme, superoxide dismutase (SOD), which was responsible to remove the ROS and to prevent cells from oxidative damages. However, POD activity and CAT activity were not enhanced at the early period of hypocotyl exposure. It was suggested that the treatment of exposing hypocotyl was not a stress severe enough to induce immediate activation of POD and CAT. In some cases, plants acclimating to stresses or released from stresses do not show enhanced activation of POD and CAT although SOD activation is confirmed. Pan et al. (2006) found that SOD and POD increased but CAT decreased when *Glycyrrhiza uralensis* plants were subjected to salt and drought stresses. In case of pigeonpea, the genotype exhibited lower accumulation of catalase (CAT) but increased activity of superoxide dismutase (SOD) and peroxidase (POD) under stressed condition (Ranjan 2011).

In the present experiment, improvements in dry mass production, photosynthetic activities by hypocotyl exposure were confirmed and attributed to osmotic adjustment. The analysis of osmotic adjustment was made just after the hypocotyl exposing treatment and showed larger differences in the related parameters between treatment and control than in the previous experiments, where osmotic analysis was made after the stimulation was released for months. Therefore, analysis in the present experiment more clearly confirmed the benefits from the osmotic adjustment. Under severe drought, osmotic adjustment is an energy and biomass consumable process. Plant growth and yield may be negatively affected in order to gain plant survival in drought stress conditions (Jones et al. 1980; Wyn-Jones and Gorham 1983; Morgan 1984). However, in the present experiment, analyses of antioxidant enzymes, plant biomass production

and physiological processes including photosynthesis and osmotic adjustment all indicted that hypocotyl exposure was just a mild stimulation without damages to plants but sufficiently induced expected benefits through the xerophytophysiological regulations. The detailed mechanisms at molecular biological levels will be examined in other experiment and discussed in the next chapter.

Chapter 10

Increases in Transcript of a Drought Responsive Gene *Gdi-15* in Relation with Accumulations of Anthocyanin and Sugars in Peanut Seedlings with Their Hypocotyls Exposed

10.1 Abstract

In the previous experiments with different types of AnM techniques, exposing the hypocotyls of peanut seedlings to light and dry air induces xerophytophysiological regulations such as increases in leaf turgor potential and improved the final pod yield. As a clear indication of the xerophytophysiological regulations, anthocyanins started to accumulate from the upper part of the hypocotyl immediately after the hypocotyl was exposed to light. In the present experiment, a drought responsive gene, *Gdi-15* was analyzed to clarify mechanisms at molecular biological level for the xerophytophysiological regulations induced by hypocotyl exposure treatment. Results of real-time PCR confirmed that transcript levels of *Gdi-15* clearly increased in the exposed hypocotyl and was proportional to anthocyanin accumulation. Amyloplasts were decreased in the exposed hypocotyl, suggesting that the carbohydrate-consumable regulation processes occurred in exposed hypocotyl and carbohydrates might be used to biosynthesis of anthocyanins and other osmolytes. However, up-regulation expression was not found in leaves and root, where the stimulation was not directly imposed. Biomass accumulation of leaves and root was not negatively affected but increased by exposing the hypocotyl. It was suggested that the up-regulation expression of the drought-responsive gene and carbohydrate-consumable regulations in the exposed hypocotyl might provide promotion messages and create positive conditions to shoot and root of the peanut seedlings.

Keywords: AnM technique, anthocyanin, *Gdi* (Groundnut Desiccation Induced gene), hypocotyl exposure, xerophytophysiology.

10.2 Introduction

In different types of AnM techniques, the key point in common in practice is exposing the hypocotyls of peanut seedlings to light and dry air to induce xerophytophysiological regulations. In all the experiments, irrespective of field and laboratory, anthocyanins started to accumulate from the upper part of the hypocotyl immediately after the hypocotyl was exposed. This phenomenon was visually observed in the peanut seedlings and confirmed by anthocyanin quantification data (Chapter 4). Biosynthesis of anthocyanins is catalyzed at the last step by UDP-glucose:flavonoid 3-O-glucosyltransferase (F3OGT) by the transfer of the glucosyl moiety from UDP-glucose to the 3-hydroxyl group of anthocyanidins, producing the first stable anthocyanin after the colorless leucoanthocyanidin molecules are converted by anthocyadin synthetase (ANS) (Holton and Cornish 1995). It was found that *Gdi-15* (Groundnut desiccation induced), *Arachis hypogaea* putative flavonol 3-o-glucosyltransferase, was a stress-responsive gene in peanut (Gopalakrishna et al. 2001). *Gdi-15* transcripts increase markedly in response to stress, shown to be up-regulated in drought conditions. Functional characterization of the desiccation-induced gene *Gdi-15* was confirmed by virus-induced gene silencing and RNAi in *Nicotiana benthamiana* and *Arabidopsis* (Senthil-Kumar et al. 2007; Senthil-Kumar et al. 2010). The flavonoid 3-O-glucosyltransferase (F3OGT) silenced tobacco plants and *Arabidopsis f3ogt* mutant showed more wilting symptoms, membrane damage and chlorophyll degradation during water deficit, signifying its function in drought tolerance in groundnut (Senthil-Kumar et al. 2007; Senthil-Kumar et al. 2010). In addition, *Arabidopsis f3ogt* null mutant exhibited altered levels of tolerance to bacterial pathogens (Senthil-Kumar et al. 2010). A hypothesis is proposed by Senthil-Kumar group that anthcyanins play important roles in drought and pathogen resistance through *F3OGT* gene regulation and make plants healthy (Senthil-Kumar and Mysore 2010). As suggested by the abovementioned research cases, anthocyanin accumulation is a defense response of plants to environmental stresses (Eryilmaz 2006; Tira-Umphon et al. 2007), and as confirmed by the previous experiments, it is clearly induced in peanut seedlings by hypocotyl exposing treatment. In addition, biosynthesis of anthocyanins is clearly related with the drought-responsive gene, *Gdi-15*. Other xerophytophysiological regulations such as accumulation of sugars and leaf turgor improvement are also found in hypocotyl exposed peanut seedlings but the related drought-responsive genes other than *Gdi-15* are not clear and complicated, if any. Therefore, in the present experiment, expression of *Gdi-15* was analyzed and the relations with anthocyanin accumulation and other osmolytes accumulation were discussed.

10.3 Materials and methods

10.3.1 Plant materials and treatments

Plant material was a Japanese peanut cultivar (*Arachis hypogaea* L. cv. Chibahandachi). Seeds of peanut were sown in the field in early May. The planting condition referred to basic AnM method in Chapter 2. In this experiment, hypocotyl exposure was started one week after seed sown, and only "n" stage was involved. The light density to which hypocotyls were exposed was more than 1200 μmol m^{-2} s^{-1} at midday.

10.3.2 Anthocyanin measurement

Fresh sample of 0.3 g hypocotyl from the upper part of the hypocotyl was soaked into 10 ml of methanol containing 1% HCl, boiled for 10 min and then incubated in darkness overnight at room temperature. The absorbance of the extracts at 530 nm was measured. One unit of the relative anthocyanin content (A) was expressed as 0.1 time of optical density (OD_{530}) per fresh weight in 10 ml extraction buffer. The anthocyanin content of cotyledons was measured in the same way. The measurement was repeated three times.

10.3.3 RNA extraction, cDNA synthesis and real-time PCR

Total RNA from leaf, cotyledons and root of the peanut seedling was extracted using RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA from hypocotyl was extracted using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. One μl of total RNA from each organ was reverse transcripted to cDNA using PrimeScript® II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The cDNA was diluted 20 times and 2 μl of the diluted cDNA was used as template in each reaction for quantitative real-time RT-PCR analysis. The cDNA was amplified using SYBR® *Premix Ex Taq*™ II (TaKaRa) on the ABI StepOne™ real time PCR system (Applied Biosystems, USA). *β*-actin gene from peanut was used as an internal standard to normalize the expression for *Gdi-15* gene. The PCR was performed as follows: 95 ˚C for 10 min, followed by 50 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min. A dissociation kinetic analysis was performed to check the specificity of the annealing. Three replications were performed for each sample. The following primer pairs were used for *Gdi-15* (AY029322) and *β*-actin (DQ873525) amplification:

Gdi-15, forward, 5'-GGTGTTCCCATGATTGC-3';

reverse, 5'-GCCTTGGTAGAAGAGCC-3';

β-actin, forward, 5'-GGTTCCACTATGTTCCCAGGCA-3';

reverse, 5'-CTTCCTCTCTGGTGGTGCTACA-3' (Chen et al. 2011).

10.3.4 Measurement of soluble sugars

The measurement of soluble sugars was according to the anthrone-sulfuric acid method (Somani et al. 1987). Carbohydrates, such as soluble sugars, are dehydrated by concentrated sulfuric acid to form furfural, which condenses with anthrone to form a

blue-green colored complex solution. Sample of 0.2 g hypocotyl or leaf of peanut seedlings was added with 5 ml distilled water, covered with parafilm and incubated in boiling water for 30 min. The extracted solution was diluted with water to set the volume to 25 ml. The reaction was as follow: 0.5 ml of sugar solution, 1.0 ml distilled water, and 0.5 ml of saturated anthrone solution (ethyl acetic saturated with anthrone) and 5 ml of concentrated sulfuric acid are added. The reaction was under boiling water for 10 min, cooled down to room temperature. The solution shows the maximum absorption at 620 nm. Glucose was used to make the standard regression curve under different content of glucose. The reaction was the same as the above mentioned. The soluble sugar content is expressed as mg per g of fresh weight.

10.3.5 Determination of proline concentration

Proline colorometric determination was conducted according to Bates et al. (Bates et al. 1973; Marin et al. 2010) based on the reaction of proline with ninhydrin. Ninhydrin reacts in particular with proline, an imine in which the amine group has cyclized with the side chain to form colored substance. Sample of about 0.3 g hypocotyl or leaf was used for proline extraction in 5 ml 3% sulfosalicylic acid solution for 10 min at 100˚C. A 1:1:1 mixture of extracted proline solution, glacial acetic acid and 2.5% acid-ninhydrin was incubated at 100˚C for 30 min. The reaction terminated in an ice bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The proline content was expressed as μg per g of fresh weight.

10.3.6 Measurements of soluble proteins

The method referred to Chapter 11 materials and methods 11.3.2.

10.3.7 Microscopic observation of anthocyanin in epidermal cells of hypocotyl

One of the thin layers of epidermal tissue was peeled off from upper part hypocotyl of the peanut seedling and transferred to a drop of water on a microscope slide.

10.3.8 Anatomical observation of hypocotyl structure

The upper part of the hypocotyl about 0.5 cm long was fixed in FAA solution (1:1:18 = formaldehyde: acetic acid: 70% ethanol) for at least 24 h. The fixed hypocotyl specimens were transferred to clearing solution (70% ethanol) for 1 h to wash off the FAA solution in avoidance of damage to plant tissue, and dehydrated was through the graded ethanol series to displace the water with a minimum of 1 hour in each of the solutions. The graded ethanol series were 70%, 85%, 95% and 100% ethanol. Then the hypocotyl specimens were transferred to ethanol:xylene solution (2:1), ethanol:xylene solution (1:1), ethanol:xylene solution (1:2) and xylene solution, each for 1 hour to displace ethanol. Pellets of paraffin were added several times in the infiltrated tissues half filled with xylene and keep at 65-70 ˚C overnight. The paraffin dissolved into xylene and slowly infiltrated the tissues with a mixture of paraffin and xylene. The next day, half of the xylene/paraffin mixture was decanted and refilled with fresh molten paraffin at 65-70˚C. Make paraffin exchange several times until the tissues were

embedded into paraffin. The paraffin embedded specimens were mounted onto a rotary microtome (Leica RM2016, Germany) and sliced to 8 μm section. Staining of deparaffinized specimens with Periodic acid-Schiff (PAS) reagent or with Safranin O/fast green was performed according to standard histological procedures to detect carbohydrates and starch (Johansen 1940; Mazia et al. 1953; Chapam 1975). Stained specimens were observed under a Nikon Microscope (Japan) with a Nikon FDX-35 camera.

10.4 Results

10.4.1 Hypocotyl exposure induced anthocyanin accumulation and up-regulated the expression of *Gdi 15* gene in hypocotyl

As hypocotyls were exposed to light by removing the soil around, anthocyanins accumulated soon from the upper part of hypocotyl to the middle part after hypocotyl exposure and reached to the peak at day 5 (Fig. 10.1 A, B). However, anthocyanins were also visible from day 3 in the upper part of hypocotyls of control seedlings (Fig. 10.1 A), although the content of anthocyanins was considered to be negligible compared to that in exposed hypocotyls (Fig. 10.1 B). This was probably attributed to the crack of soil surface through which the light irradiation like red and far-red could penetrate and also be received by the upper part of underground grown hypocotyls in control condition.

It was found that anthocyanins accumulated predominantly in epidermal cells of hypocotyls. When hypocotyls were exposed to light, anthocyanins accumulated quickly within 6 h (Fig. 10.2 B-2). Previous studies found that stomata differentiation is promoted by light in the epidermal layer of the hypocotyls, which are absent from the hypocotyls in the dark (Arnim and Deng 1996). The existing stomata might play an important role in anthocyanin production under various kinds of stresses (Kawamura et al. 2000). In the present study, it was found that anthocyanins accumulated at the beginning around the stomata and distributed like bands (Fig. 10.2 B-3). Not all the epidermal cells accumulated anthocyanins. As light was received further (eg. at day 2) by the hypocotyls, the anthocyanin-containing cells spread around and the red color was accumulated more deeply (Fig. 10.2 C-2). Interestingly, anthocyanins accumulated like a string in epidermal cells of control hypocotyls at 6 h and gradually accumulated to a narrow band at day 2, although the pigment was invisible in hypocotyls of control seedlings (Fig. 10.2 B-1 and C-1, red arrows). In addition, stomata in hypocotyls of control seedlings were found with two entire plump guard cells (Fig. 10.2 D-1) which might play the roles in transpiration and controlling water loss in hypocotyls underground. However, more than 2 days of hypocotyl exposure resulted in stunken and closed stomata (Fig. 10.2 C-2 and D-2, black arrows) which were speculated to have lost the functions in light-exposed conditions.

Gdi-15 (Groundnut Desiccation Induced) gene was found to be a stress-responsive gene that showed homology to flavonoid 3-O-glucosyltransferase involved in anthocyanin biosynthesis (Gopalakrishna et al. 2001). In the present study, real-time PCR analysis showed that *Gdi-15* transcript levels in exposed hypocotyls increased 1.5-3 folds especially within 5 days compared to control in response to light exposure (Fig. 10.1 C). In field planting condition, the light density could reach to more than 1200 μmol m^{-2} s^{-1} at midday. Hypocotyls were exposed to such strong light, which might become a light stress for the young hypocotyls that usually grew underground. These results indicated that hypocotyl exposure might be as a light stimulus leading to

the up-regulation of *Gdi-15* and anthocyanin biosynthesis which served an UV or photo-protective role in light stress.

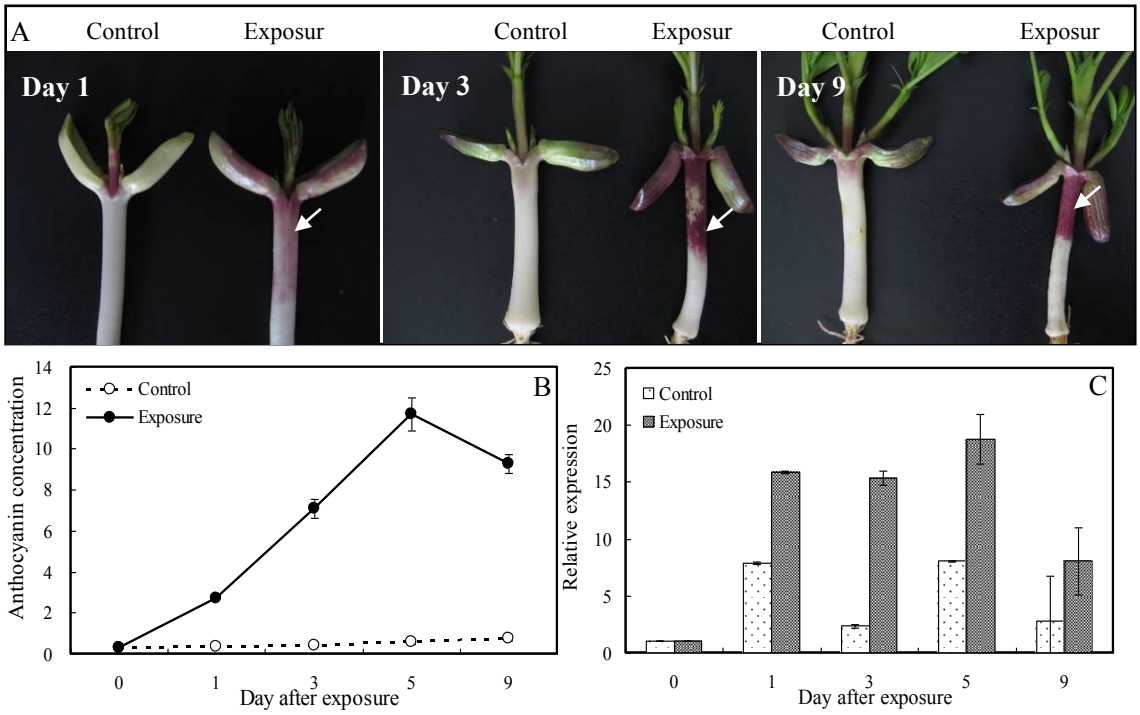

Fig. 10.1 Anthocyanin concentration in hypocotyls of peanut seedlings. A, seedlings at day 1, day 3, and day 9 after hypocotyl exposure treatment; B, anthocyanin concentration in hypocotyls; Anthocyanin concentration was expressed as OD_{530} per g^{-1} fresh weight. C, real-time PCR analysis of *Gdi-15* gene transcript levels in hypocotyls; White arrows indicate anthocyanin accumulation. The growth condition was referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Error bars represent SE values for three or four independent replicates.

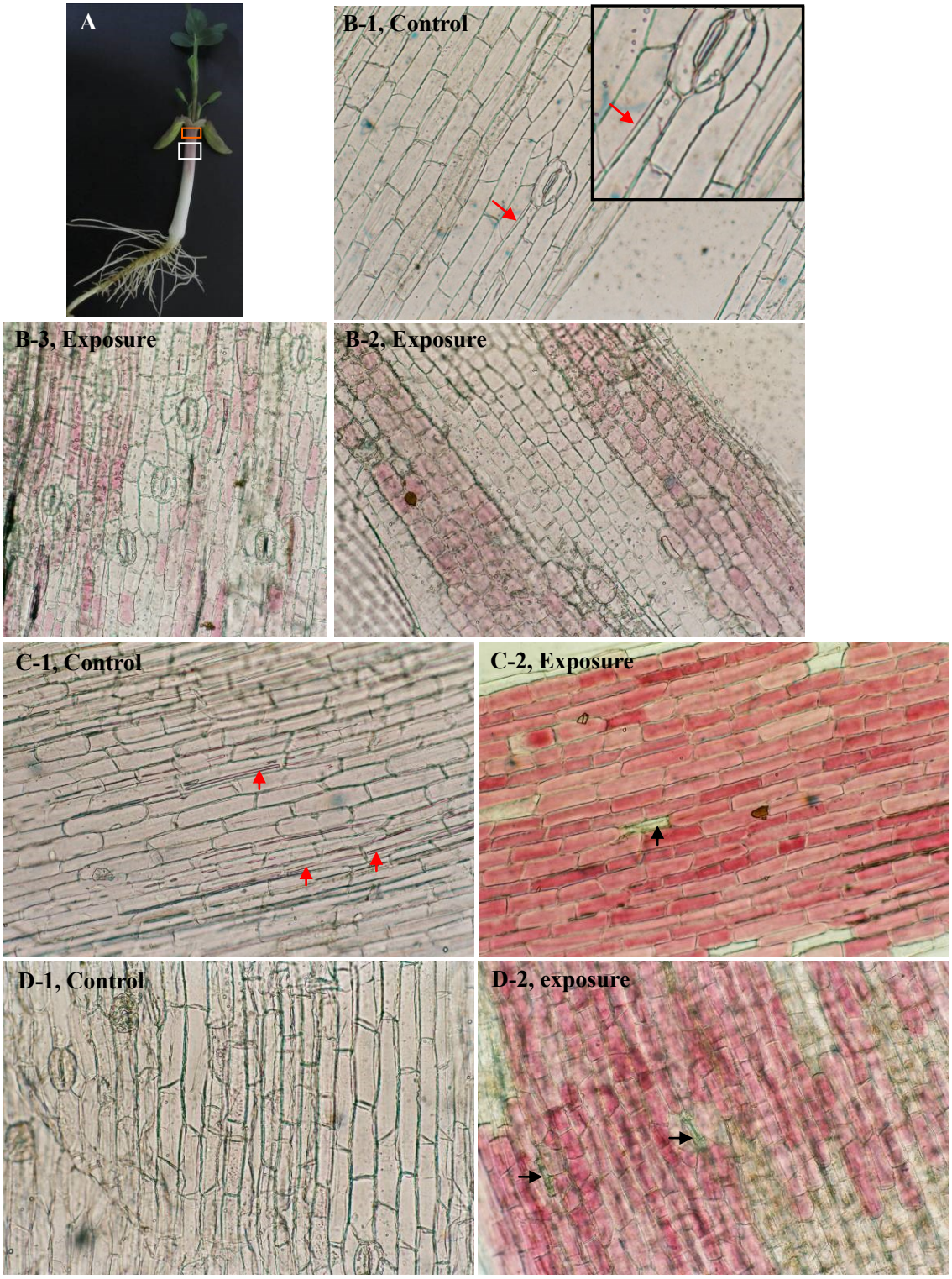

Fig. 10.2 Optical micrographs for anthocyanin accumulation in epidermal cells of hypocotyl of peanut seedling grown in field condition. A, the upper part (shown as white square) of the hypocotyl used for observation of anthocyanins; yellow square shows the part of hypocotyl just below the cotyledons. B-1, B-2 and B-3, 6 h after hypocotyl exposure. C-1 and C-2, 2 days after hypocotyl exposure. D-1 and D-2, 5 days after hypocotyl exposure. All the micrographs were taken using 200× magnification.

Red arrows, threadlike anthocyanins accumulated in epidermal cells of control hypocotyls; Black arrows, sunken and closed stomata caused by hypocotyl exposure. The planting condition referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface.

10.4.2 Hypocotyl exposure accelerated amyloplasts to turn into other forms of energy for seedling development

In the present experiment, the exposed hypocotyl was the only difference between the control seedling and the treated seedling. It was speculated that there would be some other difference except anthocyanin pigments occurring in cytological levels of hypocotyls by the light exposure. Therefore, the comparative analysis of histological structure in hypocotyls was conducted.

It was found that there was no difference in anatomical structure between the exposed hypocotyls and control hypocotyls. The anatomy typical of peanut hypocotyl includes epidermis, cortex, phloem, xylem and pith. However, when the deparaffinized sections stained with safranin O/fast green, a large amount of red particles were found accumulated in cytoplasm under 100× magnification in control hypocotyl cells but fewer in exposed hypocotyl cells especially at day 3 after hypocotyl exposure (Fig. 10.3 A, B). These red particles were found clearly under 200× magnification accumulated mainly in cortex cells around phloem and in pith cells near the xylem (Fig. 10.3 C, D). Using another staining method by Periodic acid-Schiff (PAS), these red particles also found in cytoplasm (Fig. 10.3 E). It was supposed that these red particles were amyloplasts by observation under 1000× magnification (Fig. 10.3 F).

Number of amyloplasts both per cell and per mm^2 showed no obvious difference at first 6 hours of light exposure. Since 1 day, the cells of control hypocotyls started to form large amount of amyloplasts and accumulated more at day 3 in cells of cortex near the phloem and in parenchyma cells of pith, but fewer in cells of exposed hypocotyls (Fig. 10.4 A, B). Amyloplasts are non-pigmented organelles that are responsible for the synthesis and storage of starch granules through the polymerization of glucose (Wise 2006). The results suggested that destructive consumption of carbohydrates might occur in the exposed hypocotyls. Moreover, the biosynthesis of anthocyanins in hypocotyls is a process of sugar exhaustion, which needs glycosylation by binding and transporting reactive monosaccharides. Amyloplasts in cells of exposed hypocotyls might convert this starch back into sugars when the seedlings of peanut plant need energy. In addition, amyloplasts and chloroplasts are closely related. Amyloplasts in cells of exposed hypocotyls might turn into chloroplasts; this is for instance observed when hypocotyls were exposed to light and turned green (Anstis and Northcote 1973).

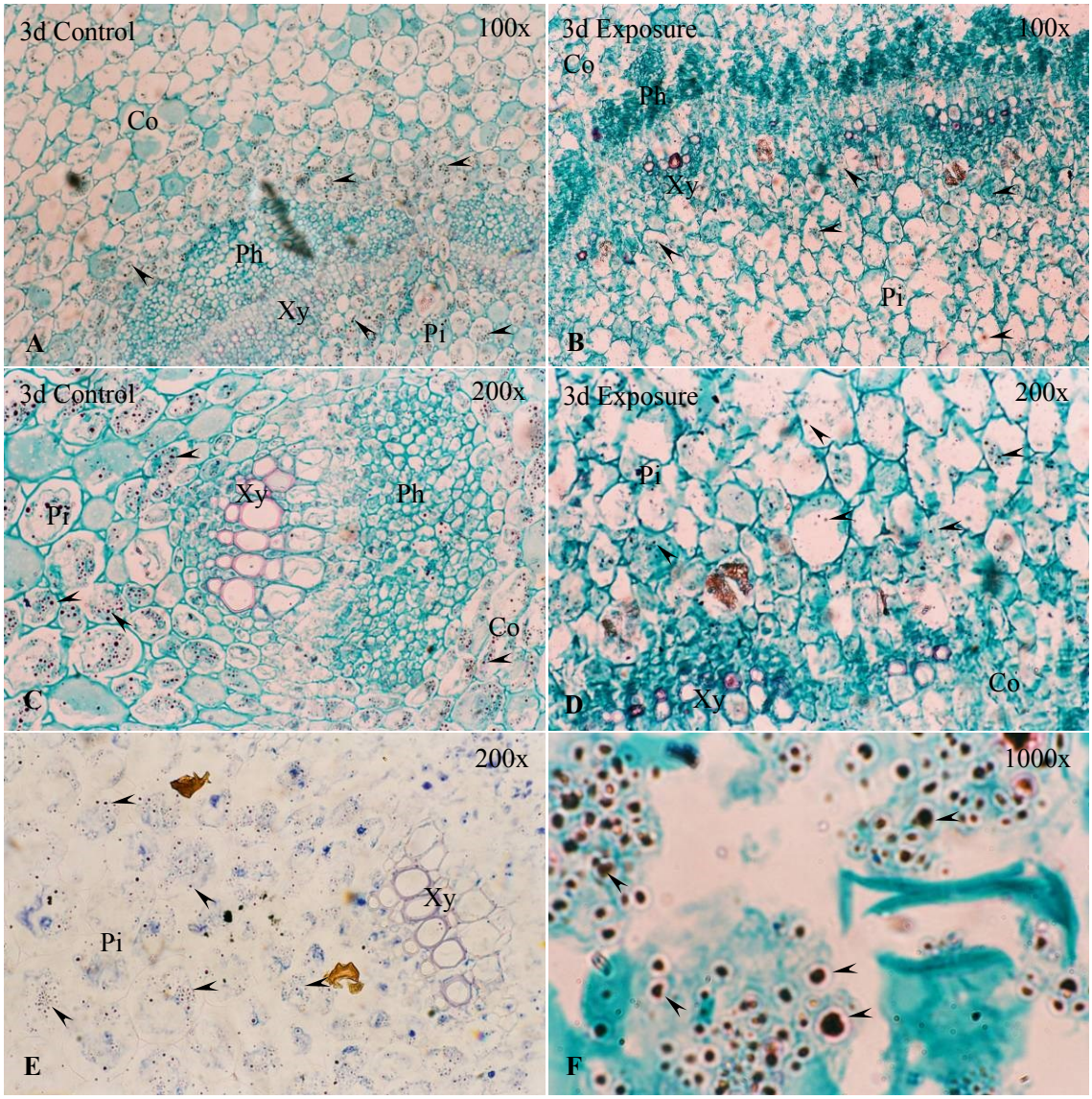

Fig. 10.3 Microscope images of amyloplasts in hypocotyl cells of peanut seedlings 3 days after hypocotyl exposure. All the sections were transverse sections. The micrographs were taken using 100× (A, B), 200× (C, D and E) and 1000× (F) magnifications. A-D and F, paraffin sections of hypocotyl using safranin O/fast green staining; E, paraffin sections of hypocotyl using Periodic acid-Schiff (PAS) staining. Black arrowheads indicate amyloplasts in hypocotyl cells shown by the red particles. The planting condition referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 µmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Co, cortex; Ph, phloem; Xy, xylem; Pi, pith.

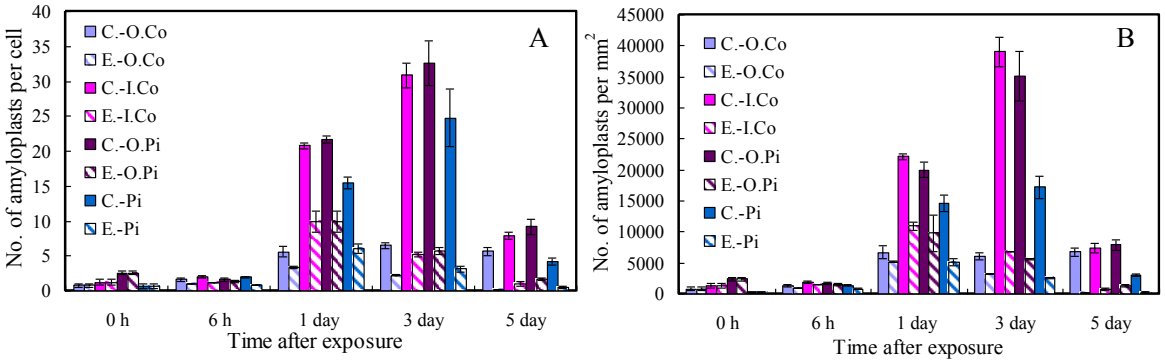

Fig. 10.4 Amyloplasts number in hypocotyl cells of peanut seedlings. The planting condition referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. C., control; E., exposure; O.Co, the outer layers of cortex; I.Co, the inner layers of cortex around the phloem; O.Pi, the outer layers of pith near the xylem; Pi, the central part of pith. Amyloplasts particles were counted in three randomly selected areas (0.005mm^2). Error bars represent SE values for three independent replicates.

10.4.3 Hypocotyl exposure down-regulated the expression of *Gdi-15* gene in root and leaf, but promoted root and leaf development

Irrespective of the involvement of *Gdi-15* in anthocyanin biosynthesis, *Gdi-15* (Groundnut desiccation induced) is a stress-responsive gene which shows up-regulated in drought conditions (Gopalakrishna et al. 2001). Unlike the expression in exposed hypocotyl, interestingly, real-time PCR analysis showed the down-regulation of *Gdi-15* gene in root and leaf where the stimulation was not directly imposed (Fig. 10.5 A, B).

Instead of inhibition by hypocotyl exposure, biomass accumulation of leaves and root was not negatively affected but increased by exposing the hypocotyl (Fig. 10.5 C, D). The growth of root was promoted even just one day exposure treatment (Fig. 10.5 C-a, b) and more marked from day 9 after hypocotyl exposure (Fig. 10.5 C-c, d). These results indicated that hypocotyl exposure did not impose stress on root and leaf of peanut seedlings, but a promotion in development. This might be probably the reason for the low expression of *Gdi-15* gene in root and leaf.

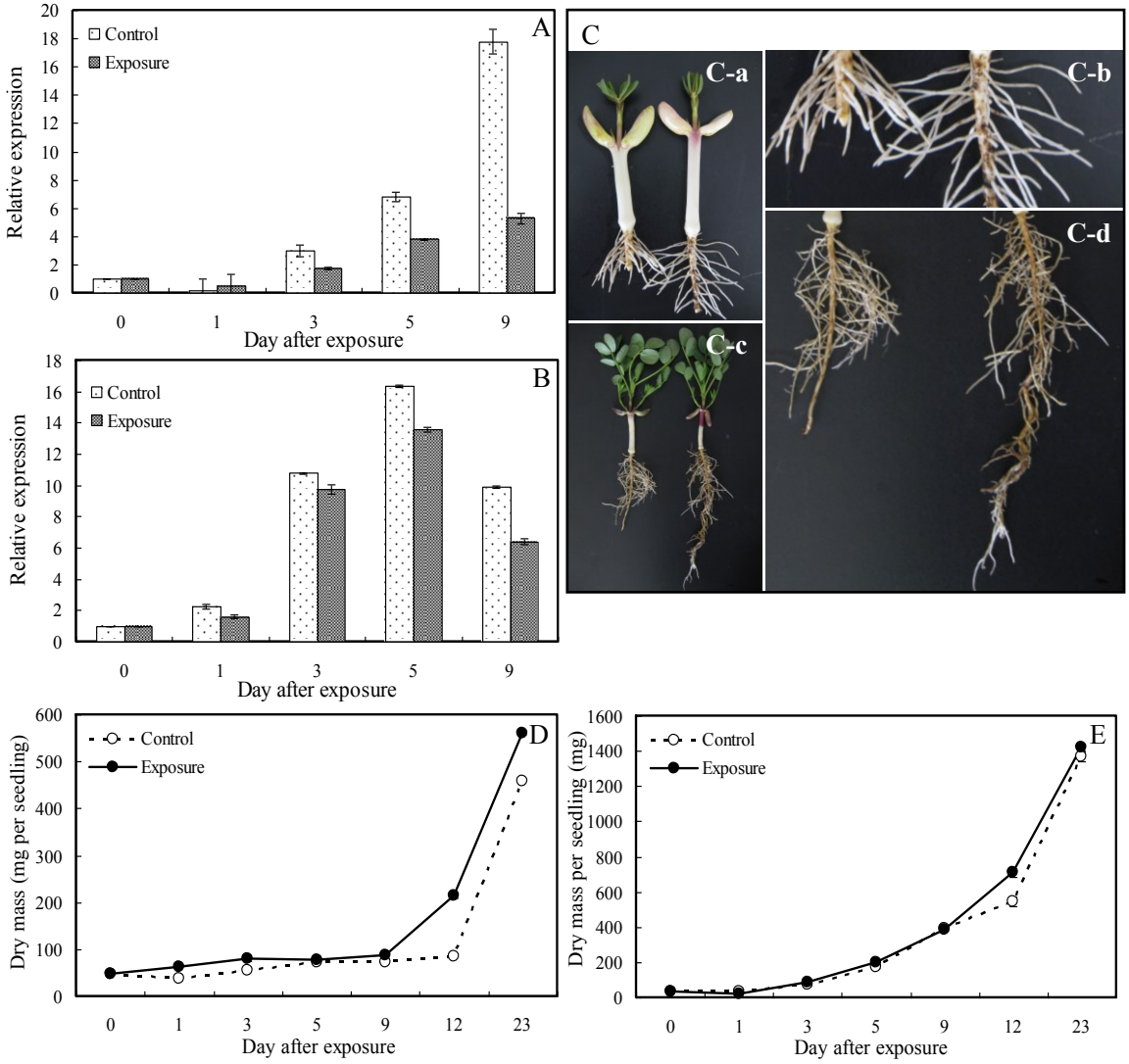

Fig. 10.5 Real-time PCR analysis of *Gdi-15* gene and biomass accumulation in root and leaf of peanut seedlings. A and B, real-time PCR analysis of *Gdi-15* gene in root and leaf, respectively; C, roots of peanut seedlings. Left, control; Right, exposure; C-a, peanut seedlings 1 day after hypocotyl exposure treatment; C-b, the magnification of root in C-a; C-c, peanut seedlings at day 9 after hypocotyl exposure treatment; C-d, the magnification of root in C-c. D and E, dry mass of root and leaf, respectively. The planting condition referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Error bars represent SE values for three or six independent replicates.

10.4.4 No difference in *Gdi-15* gene expression in cotyledon by hypocotyl exposure

In the present experiment, hypocotyl exposure completely sent out of the cotyledons to light unlike the half emergence of cotyledons in the most cases. Anthocyanin content was accumulated higher in the cotyledons of hypocotyl-exposed seedlings than in control seedlings (Fig. 10.6 A). Under the field condition in which hypocotyls were exposed to strong light, the transcript levels of *Gdi-15* gene in cotyledons increased from day 1 to day 5 although it sharply decreased at day 1 under both hypocotyl exposure and control, but it showed no obvious difference at first 3 days between hypocotyl exposed treatment and control (Fig. 10.6 B).

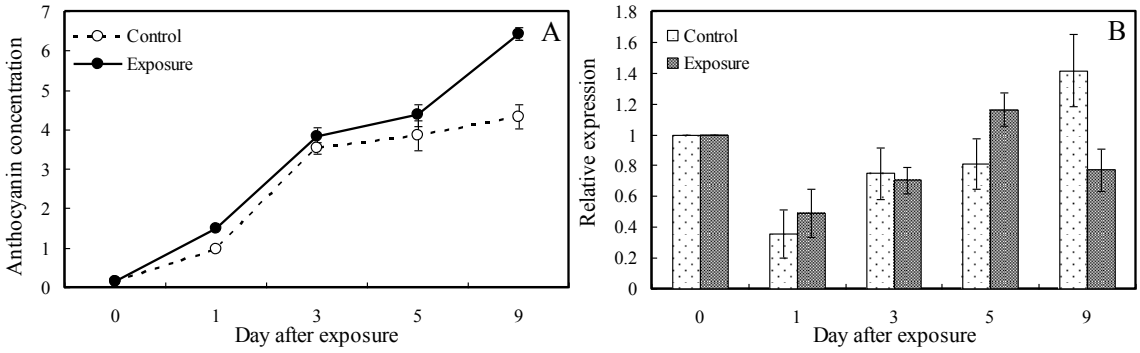

Fig. 10.6 Anthocyanin concentration (A) and real-time PCR analysis (B) of *Gdi-15* gene transcript levels in cotyledons of peanut seedlings. The planting condition referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Error bars represent SE values for three or four independent replicates.

10.4.5 Hypocotyl exposure induced the accumulation of other osmolytes

Plants accumulate different types of organic and inorganic solutes in the cytosol to lower osmotic potential thereby maintaining cell turgor (Rhodes and Samaras, 1994). The process of osmolytes accumulation such as proline, sucrose, soluble carbohydrates, glycinebetaine, and other solutes in cytoplasm under stresses is known as osmotic adjustment (Anjum et al. 2011).

Proline, the most common osmolyte, accumulates in many plant species in parallel with increased external stress and is considered a reliable biochemical marker of stress (Boscaiu et al. 2012). Proline accumulation is the first response of plants exposed to stress in order to reduce injury to cells (Anjum et al. 2011). In the present experiment, hypocotyl exposure to strong light caused constant increase of proline in hypocotyl (Fig. 10.7 A). In contrast, the proline in leaf decreased quickly to very low level followed by temporary increase from 1 to 3 days after hypocotyl exposure (Fig. 10.7 B). The accumulation of soluble proteins was increased by hypocotyl exposure both in hypocotyls and leaves (Fig. 10.7 C, D).

Soluble carbohydrates, such as sugars and polyols, are some of the compatible solutes used for osmotic adjustment and osmoprotection (Gil et al. 2011). In the present study, soluble sugars were decreased in exposed hypocotyls (Fig. 10.7 E), as confirmed by disappeared amyloplasts that might turn into sugars used to biosynthesis of anthocyanins or transfer to other energy used for seedling growth. However, hypocotyl exposure induced increase in the soluble sugars in leaves (Fig. 10.7 E, F).

The high proline and decreased soluble sugars in exposed hypocotyl implied that a stress might be perceived by hypocotyls. But hypocotyl exposure did not give rise to stress in the whole seedling instead the occurrence of osmotic adjustment by increase of osmolytes such as sugars, proline and soluble proteins in response to hypocotyl exposure.

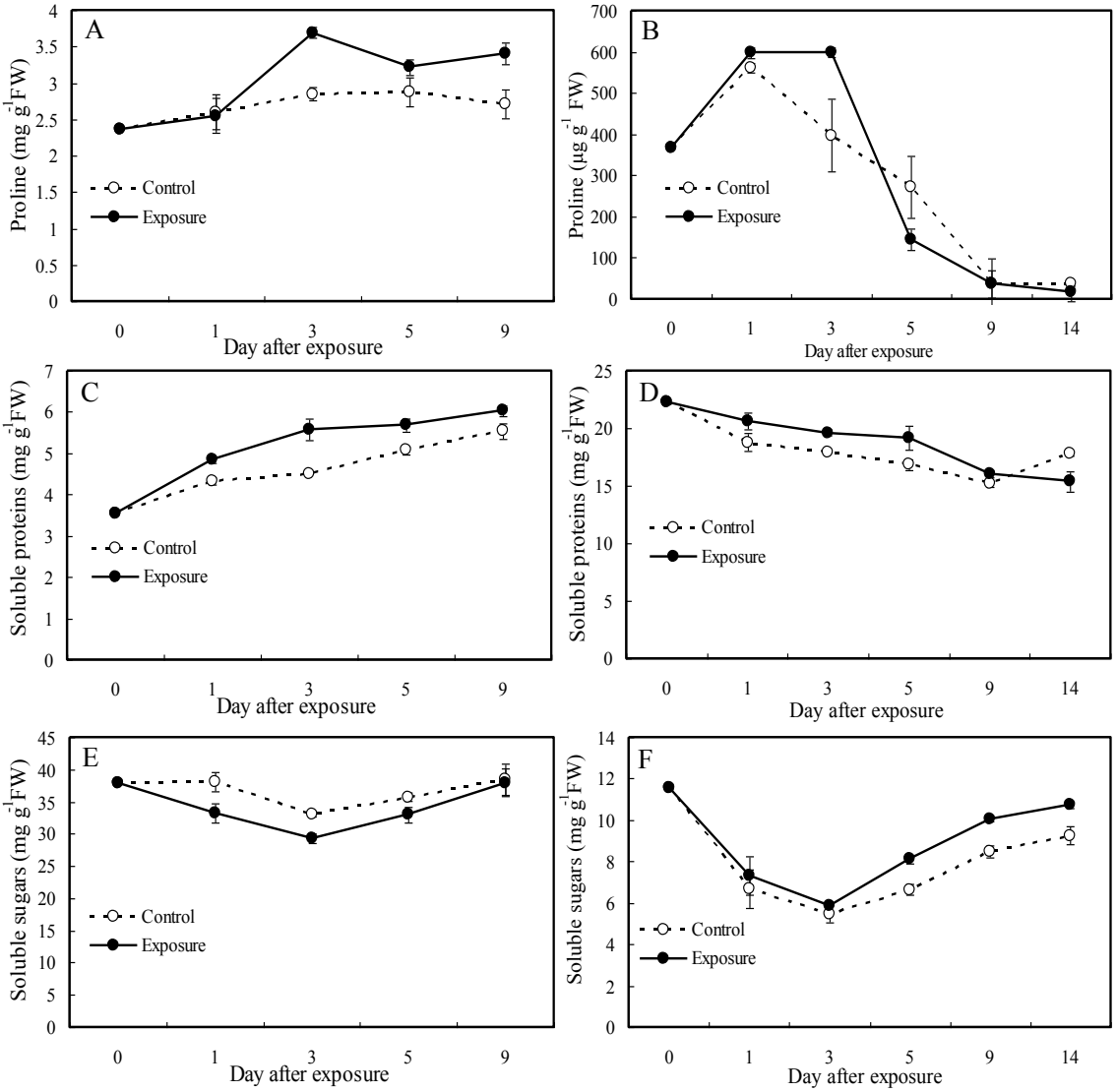

Fig. 10.7 Accumulation of other osmolytes in hypocotyl and leaf by hypocotyl exposure. A and B, proline content in hypocotyl and leaf of peanut seedling, respectively; C and D, soluble proteins in hypocotyl and leaf of peanut seedling, respectively; E and F, the soluble sugars in hypocotyl and leaf of peanut seedling, respectively. The planting condition of peanut seedlings referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Error bars represent SE values for three independent replicates.

10.4.6 Hypocotyl exposure promoted the growth and development of peanut seedlings

As hypocotyl is the only organ that perceives the stress by exposing to light, the dry mass of exposed hypocotyls deceased dramatically by consumption of carbohydrates shown by the above results, but increased rapidly later although it was still exposed to light exposure (Fig. 10.8 A).

The cotyledons of peanut are ephemeral, lasting only days after emergence. The cotyledons contain the stored food reserves of the seed. As these reserves are used up, they wither as the first true leaves take over food production for the seedling. In the present experiment, the dry mass of cotyledons decreased dramatically after 5 days exposure followed by an increase from day 3 to day 5 (Fig. 10.8 B). Hypocotyl exposure accelerated the use-up of cotyledons to supply nutrients for fast growth of the young seedlings though cotyledons could gain biomass through the probably photosynthesis.

It was found that hypocotyl exposure treatment influenced the growth and development of seedlings during hypocotyl exposure. This influence on growth was shown obviously in seedling dry mass (Fig. 10.8 C). The dry mass of seedlings was lower (although it was not obvious) in hypocotyl exposed seedlings than in control seedlings within one week after hypocotyl exposure. However, the dry mass of hypocotyl-exposed seedlings was increased higher than that of control seedlings 9 days after hypocotyl exposure. In addition, the well growth of root and leaf shown in the above results were the contributions to the promotion in seedling development by hypocotyl exposure. All these results indicated that the early negative effect by exposing hypocotyl did not last to the later growth of seedlings, but as a modest stimulus promoted the later growth and development of seedlings.

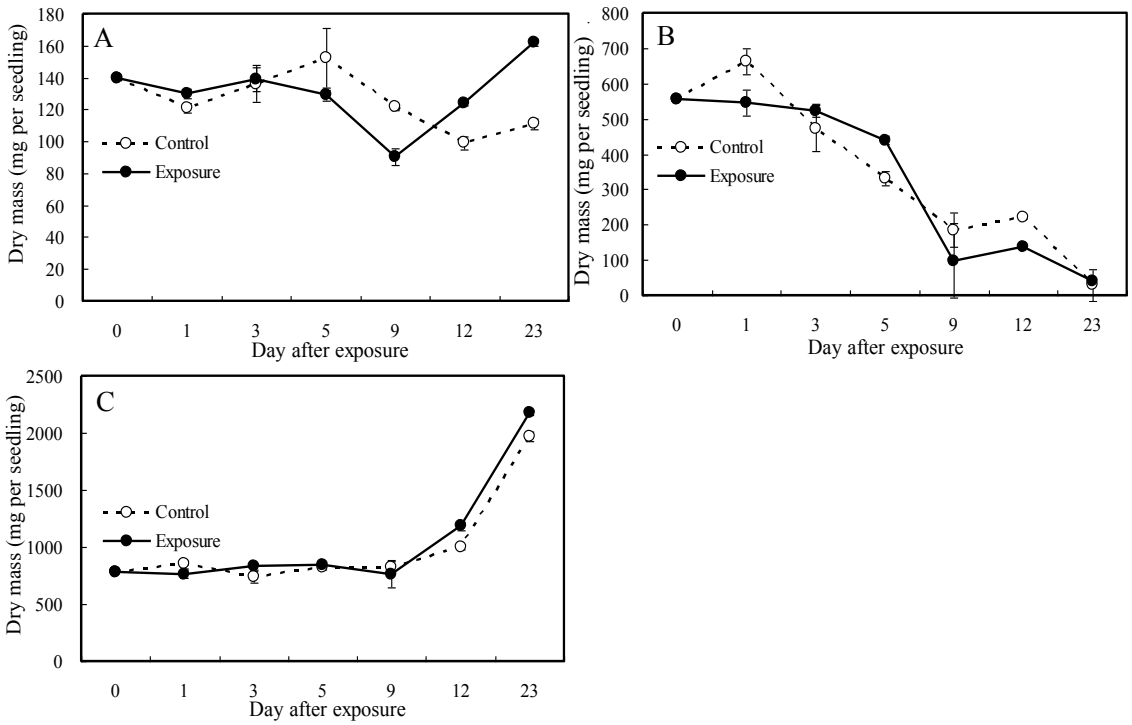

Fig. 10.8 Dry mass in hypocotyl (A), cotyledons (B) and peanut seedlings (C). The planting condition in the field referred to basic AnM method in Chapter 2. The light density at midday was more than 1200 μmol m^{-2} s^{-1}. The hypocotyl exposure started one week after seedlings just emerged with part of cotyledons coming out of the soil surface. Error bars represent SE values for three to six independent replicates.

10.5 Discussion

Soil water deficit, rhizosphere salinity, low air humidity, excess light and high temperature all cause plant desiccation and cause damages to plants. However, many plants are found having drought-responsive genes to regulate physiological processes related to desiccation resistance (Bray 1993; Ingram and Bartels 1996; Gopalakrishna et al. 2001). When a plant perceives its environmental stresses, a signal can be sent to the internal gene system, where the stress-responsive genes are activated, transcripted and expressed to enhance the physiological activities for protection and stress resistance. *Gdi-15* (Groundnut desiccation induced), *Arachis hypogaea* putative flavonol 3-o-glucosyltransferase gene (F3OGT) found in peanut, is one of these drought responsive genes (Gopalakrishna et al. 2001). Flavonoid 3-O-glucosyltransferase (F3OGT) is related with anthocyanin biosynthesis and many abiotic stress adaptations in many plants (Clive et al. 1998; Chervin et al. 2009), such as potato (Hu et al. 2011), maize (Ralston et al. 1988), *Antirrhihurn majus* (Martin et al. 1991), grape (Ford et al. 1988) and *Petunia hybrida* (Yamazaki et al. 2002). *F3OGT* is induced by a variety of environmental stimuli such as strong light, UV radiation, water stress, fungus elicitors and wounding (Tonelli et al. 1991). In the present experiment, the increased expression of *Gdi-15* gene was found in the exposed hypocotyl of the peanut seedling. The clear accumulation of anthocyanins also confirmed the enhanced expression of *Gdi-15* gene. In plant physiology, foliar anthocyanins are synthesized in response to drought, cold or saline environment and serve as osmotically active solutes to decrease leaf osmotic potential, increase water uptake and maintain leaf turgor potential in addition to functions as UV screen and free-radicals scavenger (Do and Cormier 1991; Chalker-Scott 2002). Increases in anthocyanins have been found in drought-stressed plants of many species (Spyropoulos and Mavormmatis 1978; Heikkenen et al. 1986; Balakumar et al. 1993; Allen et al. 1999; Yang et al. 2000) as well as the mechanically damaged plants (Gould et al. 2002). The color change, particularly the change in color from white to purple that the anthocyanins might be related, is a typical xerophytophysiological response (Xu 2007). Plant tissues containing anthocyanins are often resistant to drought stress although the drought resistance is not causatively linked to anthocyanin concentration. For instance, the cultivar of pepper with pretty purple or red color is more resistant to water stress than related green cultivars (Bahler et al. 1991; Tevini 1993). Ornamental shrubs with high levels of anthocyanins, such as *Cotinus* and *Photinia*, tend to be more tolerant to drought conditions (Diamantoglou et al. 1989; Knox 1989; Beeson 1992; Paine et al. 1992). Anthocyanins have antioxidant functions (Sherwin and Farrant 1998; Shan et al. 2009) by scavenging oxygen radicals and inhibiting lipid peroxidation (Tsuda et al. 1994; 1996). In the present experiment, anthocyanin accumulation in response to the hypocotyl exposure is a protective strategy against drought stress.

The decrease in amyloplast quantity and increased accumulation in osmolytes such as

sugars, proline and soluble proteins in addition to anthocyanins confirmed the occurrence of osmotic adjustment in peanut seedlings treated with hypocotyl exposure. Osmotic adjustment is a carbohydrates and energy consumable process. In the present experiment, the decrease in amyloplasts confirmed this suggestion. Amyloplasts are non-pigmented organelles found in some plant cells. They are responsible for the synthesis and storage of starch granules through the polymerization of glucose (Wise 2006). Amyloplasts can convert this starch back into sugars when the plant needs energy. Amyloplasts and chloroplasts are closely related, and amyloplasts can turn into chloroplasts; this is for instance observed when potato tubers are exposed to light and turn green (Anstis and Northcote 1973). In light-exposed shoots, plastids differentiate from proplastids or etioplasts into chloroplasts; whereas in the adjacent cells of the root, plastids differentiate instead into amyloplasts under dark conditions (Arnim and Deng 1996). In the present experiment, the carbohydrates from the amyloplasts might be used to syntheses of anthocyanins and other osmolytes, which might be transported to shoot and root to increase cell osmotic potential and maintain the turgor potential. The improved turgor potential can ensure improvements in growth of shoot and root and leaf photosynthesis.

Osmotic adjustment-caused increases in sugars were found in the present and previous experiments. Sugars also act as signaling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression. Whole-genome transcript profiling in *Arabidopsis* reveals that sugar-dependent up-regulation of the anthocyanin synthesis pathway is sucrose specific and the effect is achieved through the last few steps (DFR, LDOX and UF3GT) (Solfanelli et al. 2006). The same results have been reported in the other species (Hu et al. 2011). Hypocotyl exposure also induced increases in proline, which is one of the osmolytes, used for maintaining osmotic balance and as osmoprotectants and synthesized in plants as a general response to drought stress, although its contribution to stress-tolerance mechanisms remains unclear (Boscaiu et al. 2012). The accumulation of proline is linked to water stress, salinity and other abiotic plant stresses (Ashraf and Harris 2004; Munns and Tester 2008; Lu et al. 2009).

Accumulations of sugars, proline and soluble proteins together with the accumulation of anthocyanins were indications of the enhanced expression of the drought responsive genes. Now, it could be clearly suggested that enhance in *Gdi-15* expression was homologous to anthocyanin accumulation, but the drought responsive genes directly associated with light and accumulations of sugars and proline were not examined in the present experiment.

Gdi-15 might be considered as the representative of the drought responsive genes (Senthil-Kumar et al. 2007; Senthil-Kumar and Mysore 2010; Gopalakrishna et al. 2011). In practice, the hypocotyl is exposed but the root anchors in the sufficiently moist soil without any soil water deficit. Actually, hypocotyl exposure is not a real

stress and it is only a stimulus. The increased expression of the drought-responsive gene, *Gdi-15*, was found only in the exposed hypocotyl but not in leaves and root. During the whole experimental period, biomass accumulation in root and leaves showed increases rather than decrease. This suggested that there were no stress and the consequent damages in leaves and root even though carbohydrates were consumed in exposed hypocotyl. Nevertheless, as a false stress it successfully induced the increased expression of drought-responsive gene and the consequent xerophytophysiological regulations that might be positive to the crop. This is the key point of practices of xerophytophysiology and signal transduction in plant production, which was proposed by Xu (2007). Among practices of xerophytophysiological applications, the examples include mesocotyl exposure for sorghum plants (Xu et al. 2009d), clove exposure for garlic plants (Qin et al. 2008a), partial root-zone drying for tomato (Xu et al. 2009b) and potato (Xu et al. 2011a) crops, restricted irrigation for house tomato (Xu et al. 2011b), blue light irradiation in canopy for tomato crops (Xu et al. 2012), and seedling drying for transplanted wheat plants (Xu et al. 2011c). In most of the practices, the root of plants anchors in moist soils without real water deficit, as in case of the hypocotyl exposure of the peanut seedlings. Results of the present experiment confirmed that, as one of practices of stimulation based on the theory of xerophytophysiology, treatment of hypocotyl exposure in the AnM techniques, especially the modified AnM and seedling transplanting as the alternative of AnM, were effective in inducing enhanced expression of drought responsive genes and the expected consequences of regulations in crop production.

Chapter 11

General Discussion

Soil water deficit and salinity, low air humidity, excess light and high temperature all cause plant desiccation, which leads to injury to plants at the cellular level. However, many plants are found having drought-responsive genes to regulate physiological processes related to desiccation resistance (Bray 1993; Ingram and Bartels 1996; Gopalakrishna et al. 2001). When a plant perceives its environmental stresses, a signal can be sent to the internal gene system, where the stress-responsive genes are activated, transcripted and expressed to enhance the physiological activities for protection and stress resistance. Many research cases have been done to find these types of genes in order to breed stress-resistant varieties. This is for the purpose to grow plants under drought conditions. Many achievements have been obtained from these research cases. The detailed mechanisms have been clarified using the model plant of *Arabidopsis*. Is the knowledge on the drought resistance, especially at levels of molecular biology can be used to plant production under well watered conditions? The answer is yes.

The AnM technique in the present research is one practice to induce xerophytophysiological regulations under well watered soil conditions and make peanut plants to be healthier than usual. The AnM cultivation technique was first proposed by Shen (Shen and An 1988). The three letters, A, n and M, show the shape of the section-cross of the ridge at different stages. The practices include three steps. First, the peanut seeds are sown a little deeper than usual in the ridge to induce the extra-elongation of the hypocotyls. The second, the hypocotyls elongated more than usual are exposed to light and dry air by removing the soil away around the young seedlings just after the emergence. The third, at the middle growth stages, soils on the both sides of ridge were earthed up to welcome the late pegs. Some of the basis for advantages of the AnM technique in aspects of agronomy and botany has been clarified (Shen et al. 1987; Shen 1985; 1990; Shen and An 1988). Botanically, the elongation of peanut hypocotyl stops when the cotyledons are sent out of the soil and meet light. The hypocotyl even stops elongation and leaves the cotyledon node under the soil surface when cotyledons meet light through the soil cracks under drought conditions. Therefore, the early two branches from the cotyledon node produce pods earlier and the early pods would be infected by *Aspergillus flavus*, which produces aflatoxin and contaminates other later pods (Shen et al. 1987; Shen and An 1988; Shen 1990). The pegs and flowers can form in the soil and pollinate themselves. Therefore, it is very important to lift up the cotyledon node out of the soil. This is the key point of the step “A” to induce hypocotyl elongation. The early pods may reduce the formation of the later pods by

nutrient competition. It is also speculated that reduction of pod formation may be caused by some kind of signaling message that shows satisfaction with the existing seeds as offspring but less effort to make more seeds later. At “A” stage, seeds are intentionally sown deeper than usual in the ridge and extra-elongation of the hypocotyl is induced, which can sent the cotyledons and the cotyledon node out of the soil. At the “n” stage, the extra-elongated hypocotyl is exposed to light and dry air by removing the soil away around the hypocotyl, with the cotyledon node keeping far from the soil. The light and dry air both inhibit the penetration of the early pegs (Shen and An 1988). Consequently, the problems caused by early pods are avoided. At the “M” stage, opposite to practice of early peg control, soils from both sides of the ridge are earthed up to welcome the late developed pegs and consequently the late pegs are not too late but the early pegs are not too early, both with penetration into the soil within a relatively concentrated period (Shen 1976; Shen and An 1988). The hypocotyl exposure treatment promotes flower bud differentiation for the later pegs and helps form strong seedling. The elongation of the early pegs is controlled and the carbohydrates and nutrients are used for development of the seedlings and later pegs. In the present research, at the “M” stage, additional dressing fertilization of compound fertilizers or organic fertilizers was performed to be a good practice for supplying nutrients for development of late pegs at the same time with the practice of the soil earthing up. The advantages of the basic AnM technique in agronomy have been confirmed by experiments of the present research with a yield increase more than 20%.

However, practices of the basic AnM technique are a little complicated and not feasible for mechanization. Later, Shen et al. (1996) proposed a modified AnM method in combination with plastic-film mulching. The mulching, soil mounding and soil removing can all be performed by mechanization. Without doing so, it is not easy to practice the AnM technique in film-mulched peanut cultivation. The agronomic advantages of the modified AnM technique have been confirmed in experiments of the present research. The yield increase caused by AnM technique combined with film mulching reached as high as more than 30%. The seeded hole on the surface of plastic films on the ridge is covered with 5-7 cm high of soil mound to induce more elongation of the hypocotyls. The soil on the film is removed after the cotyledon node is sent out of the film surface. Moreover, the film mulching improved shell yield by probably controlling weeds and pests (Wang and Han 1986; Zhen et al. 2005), improving plough layer moisture and mineralization of soil nutrients (Zhen et al. 2005; Ramakrishna et al. 2006), in addition to the AnM method. The white transparent film and the black bio-degradable film mulching were compared in combination with the AnM method. In case the manufacture of the biodegradable film cannot sufficiently meet the demand in agricultural production, the white transparent one can also be a good option.

In order to further develop a more feasible AnM technique, in the present research, seedling transplanting was tried as the alternative of AnM techniques. Peanut seeds

were first sown in seedling packs and placed in dark inside an incubator. Extra-elongation of the hypocotyls induced by the dark conditions was exposed to light before transplanting. The seedlings were transplanted into field with the hypocotyls half above the soil surface. Seedling transplanting with extra elongated hypocotyls increased plant biomass and shell yield by 16% and 19% respectively. The additive and/or synergistic effects of seedling transplant in combination with AnM practice in increasing plant biomass and shell yield reached 23.7% and 33.6%, respectively. At the experiment design, the seedlings were expected for convenient hypocotyl exposition but yield increasing effect was too extravagant. Fortunately, seedling transplanting showed innegligible yield increasing effect in peanut crops. Usually, seedling transplanting is adopted in peanut production to solve the problems of bad seed germination and seedling establishment in spring when the soil temperature is low and the delay of seeding for summer rotated peanut crops after wheat harvest (Wang et al. 2002). The establishment of the summer peanut crop can be 15 day advanced and growth period is prolonged by seedling transplanting. The yield increasing effect by seedling transplanting is reported up to 12.6% to 22.2% (Wang et al. 2002). Japan is a humid country with an annual rainfall more than 1200 mm in most areas. Peanut crops are usually grown in plain field bed without ridges. In many cases, soil wet conditions adversely affect the plant growth and pod yield of peanut crop. With the AnM cultivation method, problems caused by wet soil conditions can also be solved because the ridges avoid the water accumulated in the soil and in the field. The hardening induced by hypocotyl exposure avoids the vegetative overgrowth. Therefore, in addition to the merits of the AnM treatments mentioned in the above paragraphs, the AnM method could show advantages in the specific humid conditions in Japan. In dryland conditions in countries such as China, the hardening, as shown by the osmotic adjustment and increases in turgor potential, could help the drought resistance in later dry months.

The abovementioned benefits from different types of AnM technique are described in the basis of agronomy. In all the three types of AnM technique, a key point in common is the stimulation by exposing the hypocotyl to light and dry air, which could be considered as one induction of the xerophytophysiological regulations (Xu 2007). As usual, the hypocotyl remains under moist soil without meeting light and the dry air. When the hypocotyl is exposed, the plant perceives the sudden environmental changes, produces the signal chemicals, and sends the signal to the internal gene system where some genes are activated and the related physiological and biochemical processes are regulated or adjusted. One of the main consequences is osmotic adjustment, which improves plant growth and photosynthetic activities by maintaining a higher leaf turgor potential, especially when the stimulation is released. In all the experiments of the present research, AnM techniques in different types, especially the hypocotyl exposure treatment, increased both of photosynthetic capacity and quantum use efficiency. As

usual, in a given certain range, quantum use efficiency is positively proportional to the chlorophyll content or leaf green color. In all experiments, hypocotyl exposure treatment increased the leaf green color.

The improved photosynthetic activities also reflected in less hysteresis of photosynthesis. As usual photosynthetic rate is usually higher during the second cycle of *PPF* changes than the first one (Ng and Jarvis 1980; Jones et al. 1984; Xu et al. 1994; Xu 2000). Actually, photosynthetic hysteresis shows the response or adaptability of plant to unexpected disturbances in the environment. It is usually happened to a large extent in the senescent or stressed leaves, where stomata are more difficult to open fully and the related enzymes are more difficult to be activated than those in normal plants in response to light changes (Xu et al. 1994; Xu 2000). The experiment in the present research confirmed that the improved photosynthetic activities were also reflected in less hysteresis of photosynthesis. It was concluded that less hysteresis of photosynthesis could be an indicator of physiological activities, as consequences of improved physiological activities by the hypocotyl exposure stimulation.

The improved photosynthetic activities can also be reflected in stomatal sensitivity. Experiment in the present research found that stomata in leaves of peanut plants gown with modified AnM method were more sensitive to environment changes, closing completely when adverse conditions were perceived and trying to open again when leaf water was in a relative balance. Peanut leaves in modified AnM could maintain higher stomatal conductance at the beginning and recovered to try opening stomata again but could retain the leaf water by completely closing stomata and by the low cuticular conductance at the later stages of the leaf drying period. This might be related with the physiological sensitivities and dehydration resistance in peanut leaves that was enhanced by exposing the hypocotyl in the AnM technique.

In experiments for all types of AnM techniques, osmotic adjustment was induced especially after the hypocotyl exposure and lasted to after almost one month soil earthing up. Hypocotyl exposure lowered the osmotic potential at the fully turgid status (π_{FT}). The peanut plants actively accumulated more osmotic solutes such as soluble sugars, soluble proteins in their leaf cell sap in response to the stimulation caused by hypocotyl exposure, leading to the high osmotic concentration (C_{FT}) in the leaves. Therefore, the leaf turgor potential (P) became higher, especially at the middle and later stages when the hypocotyl exposing stimulation was released and the soil was earthed up to the ridge. The high leaf turgor potential resulted in high photosynthetic activities and, as a consequence, a high final pod yield. The osmotic potential and leaf relative water content at zero turgor or the incipient plasmolysis (π_{IP} and ζ_{IP}) were lower in leaves of hypocotyl exposure treatment. The lower π_{IP} and ζ_{IP} mean that leaf turgor can be maintained to severer desiccation and are usually important for environmental stress resistance. The high leaf turgor maintenance is considered as the main one of the mechanisms for the yield increasing effect of the AnM techniques. The symplasmic

water (ζ_{sym}) was larger in leaves of hypocotyl exposed peanut plants. As usual, especially in dryland conditions, larger symplasmic water fraction or smaller apoplastic water fraction (ζ_{apo}) means higher physiological activities (Jones 1992; Xu et al. 2000). The larger cell ζ_{sym} in the hypocotyl exposed plants might be attributed to increases in osmotic substances in the symplasm inside the membrane, which might cause inward flow of water from apoplasm into symplasm. For example, as in usual, water in a cell is compartmented 73.1% in the symplasm or inside the cell membrane and 26.9% in apoplasm or the cell wall. In response to the stimulus, the hypocotyl exposed peanut plants re-compartmented the cell water 76.5% in the symplasm and 23.3% in apoplasm. As suggested by Patakas and Noitsakis (1997), the 3.4% increase in symplastic water fraction might have favored the related biochemical metabolism. The inward water flow from apoplasm to symplasm may make the symplasm more swallow and increases the cell turgor potential.

Another consequence of the xerophytophysiological regulations in peanut plants in different types of AnM plots found in the present research was the improved leaf water retention ability, which might be caused by strengthened leaf surface morphological structure. The leaf water retention ability was analyzed using the excised leaf transpiration declining curve modeled with a mathematic equation, where every constant or coefficient had a specific physiological meaning. At the first phase during the excised leaf drying period, leaves from AnM method lost more water perhaps because of a higher stomatal conductance compared with the corresponding control but soon the leaves closed their stomata and the water loss was almost only through cuticular transpiration. Leaves in AnM method closed the stomata at lower leaf water content than those from the control. This suggested that plants in AnM method were more tolerant to water deficit. After stomata closed, peanut leaves in AnM lost leaf water less than those in control, which was proportional to cuticular transpiration. As usual, cuticular transpiration would be lower if the cuticular layer was thicker or more wax was deposited into the leaf surface layer. Strengthened leaf surface structure prevented water loss from cuticular transpiration when stomata closed especially under severe water deficit conditions. Usually, this characteristic is defined as drought avoidance. In other words, a drought avoidant plant can maintain its tissue water by avoiding tissue water loss through strengthened surface barriers (Coopman et al. 2008). Drought tolerance and avoidance in pair constitute the drought resistance (Anjum et al. 2011). Even though there was no real drought and the consequent water stress in AnM treated peanut plants, the characteristics of drought tolerance and avoidance could make the plant healthier in the normal water conditions. The strengthened leaf surface structure might favor the disease resistance.

Another response of peanut plants to hypocotyl exposure stimulation is activation of antioxidant enzyme systems. The stimulation induced the production of superoxide radicals, which are not only cause damages to the cell but also act as signals to induce

stress-tolerance regulations (Jensen et al. 2008), or xerophytophysiological regulations in this case. Results of the present experiment confirmed that both the concentration of superoxide radical ($O_2\cdot^-$) and the $O_2\cdot^-$ producing rate in hypocotyl and leaves of hypocotyl-exposed peanut seedlings was significantly higher than in control. It has been noted that $O_2\cdot^-$ production appears through photosynthesis. $O_2\cdot^-$ is usually the first ROS to be generated, which may trigger the formation of more reactive ROS like OH·, and more possibly $^1O_2$, each of which may cause peroxidation to membrane lipids and cellular weakening (Bielski et al. 1983; Halliwell 2006; Gill and Tuteja 2010). The results in the present research suggested that hypocotyl exposure to light did cause a light stress for hypocotyl itself, if not for the whole plant, inducing increases in $O_2\cdot^-$ although it was just mild or false drought stimulation. Superoxide radicals are also considered as signal chemicals, which might at least collaborate with ABA together to cause signaling (Jensen et al. 2008). The oxidation, no matter severe or not, did occur in hypocotyl and leaves of the hypocotyl-exposed peanut seedlings in comparison with the control seedlings. Usually, the damage caused by $O_2\cdot^-$ can be confirmed by production of MDA, a product of ROS induced lipid peroxidation (Pan et al. 2006; Wang et al. 2011). The concentration of MDA in both hypocotyl and leaves was not higher, sometimes lower in hypocotyl-exposed seedlings than in control seedlings. It was suggested that MDA production did not really increase and there was no real or severe oxidative damages in hypocotyl-exposed peanut seedlings although the superoxide radicals were really increased. This suggested that the hypocotyl exposure treatment was just a mild stimulation instead a severe stress.

As signal chemicals, the increased superoxide radicals induced increases in protein content in both hypocotyl and leaves. Usually, mild stresses induce protein synthesis while severe stresses induce protein degradation and the same for activities of antioxidant enzymes. Activities of the antioxidant enzymes, mainly SOD, increased under mild drought stress but decreased under severe stress (Pan et al. 2006; Li et al. 2007; Li et al. 2011; Liu et al. 2011; Masoumi et al. 2011; Sun et al. 2011; Wang et al. 2011; Zhang et al. 2011), In plants adapted to stresses or just stimulated by mild stresses, protein synthesis increases and the proteolysis is reduced (Cooke et al. 1979). In the present experiment, when hypocotyl was exposed for two weeks, decreases in activity of SOD but increases in activity of POD and CA were observed, suggesting the stress caused by hypocotyl was deepening as the treatment was prolonged. In the present experiment, not only the amount of the enzymes but also the SOD activity in hypocotyl and leaves of hypocotyl-exposed peanut seedlings increased in response to stimulation treatment. It was suggested that hypocotyl exposure stimulation did induced activation of the main antioxidant enzyme, superoxide dismutase (SOD), which was responsible to remove the ROS and prevent cells from oxidative damages. However, POD activity and CAT activity were not enhanced at the early period of the treatment in exposed seedlings. It was suggested that the treatment of exposing hypocotyl was not a stress

severe enough to induce immediate activation of POD and CAT. In some cases, plants acclimated to stresses or released from stresses do not show enhanced activation of POD and CAT although SOD activation is confirmed. Pan et al. (2006) found that SOD and POD increased but CAT decreased when *Glycyrrhiza uralensis* plants were subjected to salt and drought stresses. In case of pigeonpea, the genotype exhibited lower accumulation of catalase (CAT) but increased activity of superoxide dismutase (SOD) and peroxidase (POD) under stressed condition (Ranjan 2011).

In the present experiment, improvements in dry mass production, photosynthetic activities in hypocotyl-exposed peanut plants were confirmed and attributed to osmotic adjustment induced by the hypocotyl exposing stimulation. The analysis of osmotic adjustment made just after the hypocotyl exposure treatment showed larger differences in the related parameters between treatment and control than in the previous experiments, where osmotic analysis was made after the stimulation was released for months. Therefore, analysis in the present experiment more clearly confirmed the benefits from the osmotic adjustment. Under severe drought, osmotic adjustment is an energy and biomass consumable process and plant growth and yield may be negatively affected in order to gain plant survival in drought stress conditions (Jones et al. 1980; Wyn-Jones and Gorham 1983; Morgan 1984). However, in the present experiment, analyses of antioxidant enzymes, plant biomass production and physiological processes including photosynthesis and osmotic adjustment all indicted that hypocotyl exposure was just a mild stimulation without damages to plants but sufficiently induced expected benefits through the xerophytophysiological regulations.

The detailed mechanisms at molecular biological levels were examined. Many plants are found having drought-responsive genes (Bray 1993; Ingram and Bartels 1996; Gopalakrishna et al. 2001). These genes are induced by a variety of environmental stimuli such as strong light, UV radiation, water stress, fungus elicitors and wounding (Tonelli et al. 1991). The drought-resistant varieties express drought responsive gene stronger than others under drought conditions. In the present research, the drought induced gene, *Gdi-15* (groundnut desiccation induced), was analyzed and the expression was found being enhanced by hypocotyl exposure stimulation, which was accountable for increases in anthocyanins and other osmotically active substances such as sugars, proline and soluble proteins. Accumulation of anthocyanins was observed visually in hypocotyls of the young seedlings soon after the hypocotyl exposure started. This was just one of the indications of the xerophytophysiological responses.

Actually, the anthocyanin accumulation is accompanied by accumulations of sugars, amino acids and soluble proteins as well as the production of superoxide radical and activation of antioxidant enzymes. All the consequences of the xerophytophysiological responses collaborate together to make the crop healthier through their individual function in plant growth and development. For example, anthocyanins are synthesized in response to drought, strong radiation, cold or saline environment and serve as

osmotically active solutes to decrease leaf osmotic potential, increase water uptake and maintain leaf turgor potential in addition to functions as UV screen and free-radicals scavenger (Chalker-Scott 2002). Increases in anthocyanins are found in plants of many species under drought conditions (Spyropoulos and Mavormmatis 1978; Heikkenen et al. 1986; Balakumar et al. 1993; Allen et al. 1999; Yang et al. 2000). Plant tissues containing anthocyanins are often resistant to drought stress although the drought resistance is not causatively linked to anthocyanin concentration. For instance, the cultivar of pepper with pretty purple or red color is more resistant to water stress than related green cultivars (Bahler et al. 1991; Tevini 1993). Ornamental shrubs with high levels of anthocyanins, such as *Cotinus* and *Photinia*, tend to be more tolerant to drought conditions (Diamantoglou et al. 1989; Knox 1989; Beeson 1992; Paine 1992). Anthocyanins have antioxidant functions (Sherwin and Farrant 1998) by scavenging oxygen radicals and inhibiting lipid peroxidation (Tsuda et al. 1994; 1996). Active accumulation of sugars and other osmolytes including anthocyanins, soluble protein and amino acids in the hypocotyl exposed peanut plants was the consequence of osmotic adjustment, which might be attributed together with anthocyanin accumulation to the activation and expression of the *Gdi-15* gene. The *Gdi-15* gene in hypocotyl, where the exposing stimulation was directly imposed, was confirmed to be activated, showing an up-regulation expression induced by the hypocotyl exposure. However, the same gene in leaves and root of the same hypocotyl exposed peanut seedlings was not activated or showed expression of a little down-regulation. *Gdi-15* gene is suggested as one of the drought-responsive and directly related with biosynthesis of anthocyanins, which accumulated in the hypocotyl immediately in response to the exposing stimulation. The stimulation responsive accumulation of anthocyanins could not be found in leaves and root. This result was consistent with the expression of *Gdi-15* gene.

The microscopic observation showed that amyloplasts were less in the exposed hypocotyl than in the control. This suggested that destructive consumption of carbohydrates occurred in the exposed hypocotyl even though no substantial damage was accompanied. Many research cases have suggested that osmotic adjustment or drought-responsive regulation is energy and biomass cost process (Subbarao and Johansen 2002). Activated expression of drought responsive genes and consequent osmotic adjustment, shown by the anthocyanin accumulation, concentration increase of sugars, amino acid and soluble proteins as well as the starch consumption (fewer amyloplasts), in the hypocotyl might provide with protection and improved conditions to the leaves and root. This was confirmed by the biomass production in leaves and root, which was not depressed instead increased. This was also consistent with the expression of the *Gdi-15* gene. In overall, hypocotyl exposure as a stimulation did induce the up-expression of the drought responsive gene, *Gdi-15*, and the consequent osmotic adjustment and anthocyanin accumulation in the exposed hypocotyl but causes no damage, or no real stress to the whole plant. This is the key point of applications of

xerophytophysiology and signal transduction in plant production, with false, mild or temporary drought stimulation to induce positive regulations. This was also confirmed by the leaf turgor maintenance and the consequent improvements in photosynthesis, plant growth, and the final shell yield in field experiments with different types of AnM techniques in the present research.

Because the root system of the young peanut seedling anchored in the moist soil, there is no real drought or water stress to the plant. Even though there is a real stress, some very beneficial effects can be expected from the stimuli such as mild or partial drought, water deficit, radiations and low humidity (Turner 1990; Xu 2007). Nevertheless, the sudden hypocotyl exposure to the light and dry air is a drought-like stimulation. In similar ways, other stimuli have been used to induce xerophytophysiological regulations in many plants. The examples include mesocotyl exposure for sorghum plants (Xu et al. 2009d), clove exposure for garlic plants (Qin et al. 2008a), partial root-zone drying (Dry 1997; Sadras 2009; Xu et al. 2009b) and potato (Xu et al. 2011a) crops, restricted irrigation (Dodd 2009; Xu et al. 2010), UV or blue light irradiation to peach fruit during the maturity (Rapparini 1999), and seedling drying for transplanted wheat plants (Xu et al. 2011c). Even in hydroponic culture of crops such as tomato, moderate salinity can induce xerophytophysiological regulations. The expected xerophytophysiological regulations including increases in sugars, vitamin C, organic acids and other flavors, improved fruit color, strengthened disease and pest resistance and the improved final yield. In abovementioned applications of xerophytophysiology, crops are all grown under moderate water condition without real stresses, the stimuli are just used as signal starters to induce the expected regulations, and drought resistance, even if any, is not the objective. As reported by scientists at molecular biological levels, plants are intelligent to perceive changes in environmental conditions, such as drought characterized by high UV radiation, soil water deficit, low humidity and salinity and extreme temperature, and then to transduce the signals to the internals, which induce the related gene expression and activate metabolisms related with physiological and morphological regulations or adjustments in conferring stress tolerance (Jensen et al. 1996; Mulligan et al. 1997; Smith and Gallon 2001; Davis 2004). In most of the research cases, the model plant, *Arabidopsis*, is used and few crop plants have been involved in the molecular biological research (Walley and Dehesh 2010). Scientists have always in passive positions put emphases on breeding varieties of higher resistance to the adverse environmental conditions. However, the very benefits can be induced in an active position from xerophytophysiological mechanisms by giving plants a mild or even a false drought stimulus to make the plants healthier and stronger (Turner 1990). Therefore, results of the present research perfectly confirmed that as one of practices of stimulation based on the theory of xerophytophysiology, the AnM techniques, especially the modified AnM as well as transplanting seedling with extra-elongated hypocotyls as the alternative of AnM practice, were effective in

inducing drought responsive genes and expected consequences of regulations in crop production.

General Summary

The AnM technique has been adopted in peanut production and proved effective in improvements of yield and disease resistance. However, the detailed mechanisms for the AnM technique have not been clarified. Therefore, in the present research, several experiments were carried out and biological fundamentals for the growth and yield improvement were examined in addition to confirmation of the agronomical advantages. Moreover, experiments were also conducted to confirm the feasibility of a modified AnM in combination with film mulching and an alternative by transplanting seedlings with extra-elongated hypocotyls, which were suggested easily practiced with mechanization.

The AnM practices included three steps. The three letters, A, n and M, showed the shapes of the section-cross of the ridge at the three steps of different growth stages of the peanut crop. First, the peanut seeds were sown a little deeper than usual, about 8 cm, in the ridge to induce the extra-elongation of the hypocotyl. When the seeds were sown, the cross-section of the ridge looked like the letter “A”. The second, the hypocotyls elongated more than usual were exposed to light and dry air by removing the soil away around the young seedlings just after the emergence. At this time, the cross-section of the ridge looked like the letter “n”. The third, at the middle growth stages, soils on the both sides of ridge were earthed up to welcome the late pegs. At this time, the cross-section of the ridge looked like the letter “M”.

Botanically, the elongation of peanut hypocotyl stops when the cotyledons are sent out of the soil and meet light. However, in most cases, the hypocotyl even stops elongation and leaves the cotyledon node under the soil surface when cotyledons meet light through the soil cracks. Therefore, the flowers on the early two branches from the cotyledon nodes pollinate themselves even under soil surface and produce pods earlier and the early pods compete for nutrition with the young seedlings. Therefore, the key point of “A” step was to lift up the cotyledon nodes out of the soil. At “n” step, soil around the seedling is removed and hypocotyl is exposed to light and dry air. This practice makes early pegs farther from the soil surface and thus the early formation of pods is avoided. Actually, exposing the hypocotyl is the key point of the “n” step and is also the key point of the whole AnM techniques.

In practice the modified AnM, the seeded hole on the surface of plastic film on the ridge is covered with 5-7 cm high of soil mound to induce more elongation of the hypocotyls. The soil on the film is removed after the cotyledon node is sent out of the film surface with the extra-elongated hypocotyl exposed to light and dry air. As an alternative of AnM technique more easily practiced, transplanting of seedlings was tried. Peanut seeds were first sown in seedling packs and placed in dark inside an incubator, where extra-elongation of the hypocotyls was induced. When the hypocotyls elongated

to about 5 cm long, seedling packs were moved to a lighting growth chamber. The seedlings were transplanted into the field with the hypocotyls half above the soil surface.

Agronomically, all the three types of AnM practices improved plant biomass production and final shell yield with the disease resistance also increased. The yield increment was 19.2%, 16.7% and 18.9%, respectively, for the basic, modified and alternative AnM techniques. The additive and/or synergistic yield increment reached 71.2% for the modified AnM in combination with film mulching and 33.6% for AnM treatment in addition to seedling transplanting. The bio-degradable black film was better than the transparent film because the former depressed weeds and promoted soil nutrient mineralization more effectively.

Physiologically, all the three types of AnM techniques induced osmotic adjustment, which improved photosynthetic activities by maintaining a higher leaf turgor potential, especially after the hypocotyl exposing stimulation is released. The improved photosynthetic activities also reflected in less hysteresis of photosynthesis and more sensitive stomata oscillation. In the leaves of the hypocotyl-exposed peanut seedlings, stomata closed more completely when water shortage was perceived and tried to open again when leaf water was in a relative balance. The osmotic potential and leaf relative water content at zero turgor (π_{IP} and ζ_{IP}) were lower in leaves of peanut plants with hypocotyl exposure treatment, suggesting that leaf turgor could be maintained to severer desiccation and contribute to stress resistance. Increased cell osmotic concentration might ensure the inward flow of water from apoplast to symplast and consequently the symplastic water fraction (ζ_{sym}) was larger in leaves of hypocotyl-exposed peanut plants, which might ensure, at least in part, the higher biochemical and physiological activities.

The stimulation of exposing hypocotyl to light at the “n” stage induced the production of superoxide radicals ($O_2\cdot^-$) and the $O_2\cdot^-$ producing rate in hypocotyls and leaves of hypocotyl-exposed peanut seedlings was significantly higher than in control. The concentration of MDA in both hypocotyl and leaf was not higher, sometimes lower in hypocotyl-exposed seedlings than in control seedlings. No increase in MDA suggested that there was no real or severe oxidative damages occurring in hypocotyl exposed peanut seedlings although the superoxide radicals were really increased. This suggested that the hypocotyl exposure treatment was just a mild stimulation instead a severe stress. The SOD activity in hypocotyls and leaves of hypocotyl-exposed peanut seedlings increased in response to the stimulation of hypocotyl exposure but POD and CAT activity were not enhanced at the early period of the exposure treatment. It was suggested that exposing hypocotyl was not a stress severe enough to induce immediate activation of POD and CAT.

Soon after the hypocotyl exposure started, anthocyanin accumulation was observed visually in hypocotyls of the young seedlings. The anthocyanin accumulation is

accompanied by accumulations of soluble sugars, soluble proteins as well as the production of superoxide radicals and activation of antioxidant enzymes. The microscopic observation showed that amyloplasts were fewer in the exposed hypocotyls than in the hypocotyls grown underground. Destructive consumption of carbohydrates might occur and turn into sugar or other form of energy in the exposed hypocotyls since osmotic adjustment as well as anthocyanin biosynthesis was an energy consumable process, which provided better condition for growth of shoot and root, where biomass production was improved. It is suggested that all the consequences of the xerophytophysiological responses collaborated together to make the crop healthier through their individual function in plant growth and development.

Gdi-15 (Groundnut desiccation induced) gene is a stress-responsive gene in peanut plant. It is *Arachis hypogaea* putative flavonol 3-o-glucosyltransferase which involves in the last step of anthocyanin biosynthesis. Transcript level of *Gdi-15* gene in hypocotyl, where the exposing stimulation was directly imposed, was enhanced showing an up-regulation expression induced by the hypocotyl exposure, which was consistent with increased accumulation of anthocyanins and other osmotically active substances such as sugars, proline and soluble proteins. However, the transcript levels of *Gdi-15* gene in leaves and root of hypocotyl-exposed peanut seedlings were not activated or showed expression of a little down-regulation.

In overall, hypocotyl exposure as a stimulation did induce the up-expression of the drought responsive gene, *Gdi-15*, and the consequent osmotic adjustment and anthocyanin accumulation but caused no damage to the whole plant. This is the key point of applications of xerophytophysiology and signal transduction in plant production, with false, mild or temporary drought stimulation to induce positive regulations. In conclusion, as one of practices on the theory of xerophytophysiology, the AnM techniques, especially the modified AnM as well as transplanting seedling with extra-elongated hypocotyls as the alternative of AnM practice, were effective in inducing drought responsive genes and expected positive regulations in crop production.

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