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Establishment of a Tissue Culture System of *Populus euphratica* Oliv.

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Introduction

Desertification caused by complex interplay of both artificial and natural factors is a severe environmental problem in China (Fullen, 1994). Reforestation is thought to be the most effective way to prevent desertification. Poplars are common tree species used for the reforestation of such desertified land in China. *Populus euphratica* Oliv., a species belonging to Sect. Turanga, has its distribution mainly in semiarid areas from Northwest China to the west as far as Spain and Western Morocco (Browicz, 1977). While *P. euphratica* is not a reforestation material in China, it has high tolerance for drought, salinity and alkalinity (Kang *et al.*, 1995; Kang *et al.*, 1996a; Ma *et al.*, 1997; Wang *et al.*, 1997). Hence it is thought that *P. euphratica* is not only a suitable species for reforestation in desertificated area (Wang *et al.*, 1996), but an important gene resource to develop new cultivars of poplar which are tolerant to drought.

Since artificial crossing between *P. euphratica* and other poplar species is rather difficult (FAO, 1979), biotechnology such as genetic engineering and somatic hybridization would be effective to create new cultivars having higher tolerance to drought. For this purpose, it is necessary to establish a standard method for tissue culture of this species. While Kang *et al.* (1996a, b) and others reported on *in vitro* evaluation of tolerance and plant regeneration from leaf protoplast of *P. euphratica*, there is no report on the detailed technique of *in vitro* propagation of the species.

In this study, we established a standard protocol for *in vitro* propagation of *P. euphratica* by axillary bud culture feasible for *in vitro* evaluation of tolerance and for creation of new poplar cultivars.

Materials and Methods

Stem segments, about 5 mm in length and 3 mm in diameter, with axillary bud were cut from newly developed branches of four-month-old *P. euphratica* stocks about 70 cm tall grown with hydroponics in a greenhouse. They were washed with 0.1% (v/v) neutral detergent for 1 min and rinsed by sterile water. For surface sterilization, they were immersed in 70% (v/v) ethanol for 1 min, in 1% (v/v) antiformin for 3 min and in 3% (v/v) hydrogen peroxide for 3 min, successively. After cutting the basal end of stem segment slightly, they were cultured on MS medium (Murashige and Skoog, 1962) or 1/2MS medium containing 2% sucrose and solidified by 0.8% agar. In 1/2 MS medium, major inorganic components was reduced to a half of the MS medium. They were supplemented with 0, 0.25, 0.5 and 1.0 mg/l of BAP (6-benzylaminopurine) and 0 and 0.05 mg/l of NAA (1-naphtylacetic acid) in combination (Table 1).

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Table 1. Effect of basal medium and hormonal condition of axillaly buds from *Populus euphratica**

Medium	Hormone concentration		No. of explants	Mean no. of elongated shoots** (no.±S.E.)	Mean length of elongated shoots** (mm±S.E.)
	NAA (mg/l)	BAP (mg/l)			
MS	0	0	8	0	0
	0	0.25	7	1.7±0.5	13.4±4.7
	0	0.50	7	3.1±0.8	14.6±4.7
	0	1.00	10	2.3±0.4	6.6±1.1
	0.05	0	7	0	0
	0.05	0.25	9	1.8±0.3	20.1±5.3
	0.05	0.50	8	3.1±0.6	12.4±3.0
	0.05	1.00	10	2.6±0.8	10.4±2.5
1/2MS	0	0	9	0	0
	0	0.25	7	2.3±0.7	5.0±1.0
	0	0.50	8	2.1±0.6	3.3±0.4
	0	1.00	7	1.4±0.3	4.1±1.3
	0.05	0	9	0	0
	0.05	0.25	8	2.6±0.6	6.5±2.1
	0.05	0.50	8	2.1±0.5	4.4±1.3
	0.50	1.00	8	1.8±0.3	1.9±0.3

* Data was taken after 27 days of culture.
** Contaminated samples were not included.

Table 2. Effect of NAA and BAP concentration in 1/2MS on new shoot formation in the subculture of *in vitro* propagated shoot of *Populus euphratica**

Hormone concentration		No. of samples	Mean no. of new shoots (no.±S.E.)	Mean length of original shoots** (mm±S.E.)	Ratio of vitrificated shoot (%)	Ratio of rooted shoot (%)
NAA (mg/l)	BAP (mg/l)					
0	0	10	0	9.4±0.9	0	40
0	0.1	10	3.1±0.5	19.8±0.6	30	0
0	0.2	10	3.1±0.3	19.4±1.5	20	0
0	0.4	10	2.3±0.5	20.9±1.7	40	0
0	0.8	10	2.3±0.4	18.6±2.1	50	0
0.002	0	10	0.6±0.3	10.8±0.7	50	40
0.002	0.1	10	1.8±0.6	18.1±1.8	80	0
0.002	0.2	10	1.8±0.4	19.8±1.5	80	0
0.02	0	10	1.2±0.4	11.3±0.8	70	80
0.02	0.1	10	1.7±0.5	16.0±0.8	90	0
0.02	0.2	10	2.2±0.7	17.6±0.9	90	0

* Data was taken after 4 weeks of culture.
** Data does not include length of new shoots.

Every medium used in this study was adjusted their pH to 5.8. About 10 ml of medium was poured into culture tubes (25×120 mm) and autoclaved at 120℃ for 20 min. The cultures were kept in a growth chamber at 25℃ under illumination of 200 μmol m^{-2} s^{-1} (400–700 nm) from fluorescent tubes for 16 h photoperiod throughout the experiment.

Elongated adventitious shoots, more than 5 mm in length, obtained by primary culture were cut from explants and subcultured on 1/2MS containing 0.8 mg/l of BAP, which had been used as a common medium for poplar shoot subculture in our laboratory. Shoots elongated to about 5 mm long by the subculture were harvested and used for studying effective hormonal conditions for multiple shoot formation. They were transplanted into 1/2MS supplemented with 0, 0.002 and 0.02 mg/l of NAA and 0, 0.1, 0.2, 0.4 and 0.8 mg/l of BAP in combination as shown in Table 2. At the same time, they were transplanted into 1/2MS supplemented with 0, 0.01, 0.02 and 0.04 mg/l NAA to determine the effective condition for rooting.

The rooted shoots were subsequently transferred to the pots filled with vermiculite. They were maintained under high relative humidity (85–95%) for one week, and the humidity was lowered gradually to ambient moisture level during additional two weeks for hardening. They were kept under illumination of 200 μmol m^{-2} s^{-1} (400–700 nm) for 16 h photoperiod at 25℃. Acclimatized plantlets were transplanted to potting soil and grown in a green house.

Result and Discussion

After four weeks of the primary culture, approximately 80% of explants were free from contamination. Thus we concluded the procedure used here was effective for surface sterilization of *P. euphratica*.

Axillary buds started to sprout after 11 days of the primery culture and formed multiple shoots after 27 days of culture in the presence of BAP (Table 1). A cluster of buds was formed at the base of multiple shoots especially on MS containing 1 mg/l of BAP and 0.05 mg/l of NAA. However, shoot elongation was not remarkable on the media without BAP. While maximum number of elongated shoots was attained on MS containing 0.5 mg/l of BAP irrespective of NAA concentration, the shoots were longer on the medium

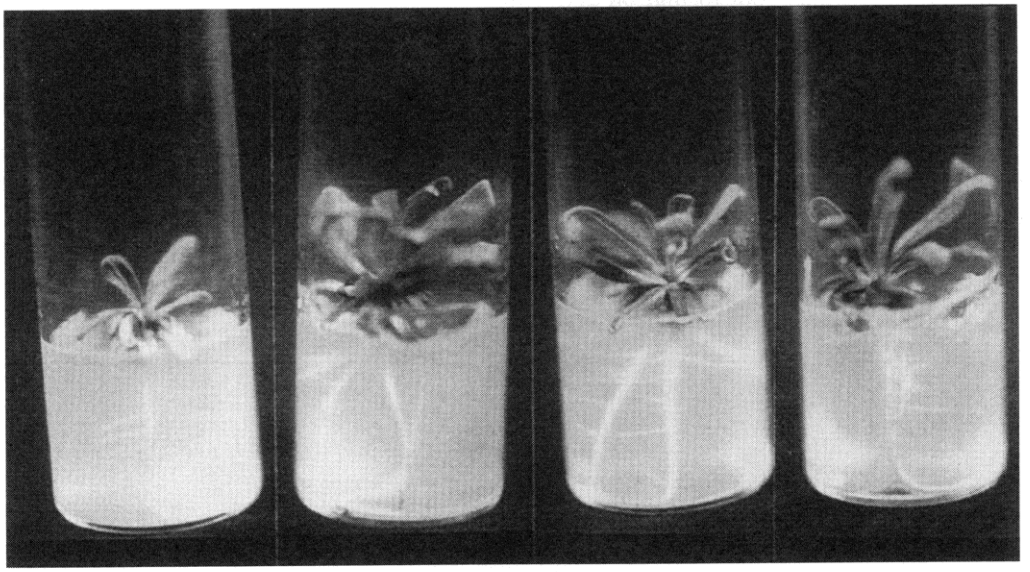

Fig. 1. Rooted shoots of *Populus euphratica* cultured on 1/2MS with different concentrations of NAA for two weeks after transplantation to the media. NAA concentrations were 0, 0.01, 0.02 and 0.04 mg/l from left to right. Diameters of tubes are 20 mm.

containing 0.05 mg/l of NAA than on the medium without NAA. Among 1/2 MS media, both number of elongated shoots and the mean length of elongated shoots were maximal on the media containing 0.25 mg/l BAP and 0.05 mg/l NAA. Mean length of shoots was longer on MS than on 1/2MS at every BAP concentration, but leaves turned yellow and died within 40 days of culture on MS (data not shown). Then we selected 1/2MS as a basal medium for the culture of *P. euphratica*. In conclusion, 1/2MS with 0.25 mg/l of BAP alone or in combination with 0.05 mg/l of NAA was the best for the primary culture of *P. euphratica*.

Shoot growth and other responses of explants after four weeks of subculture are shown in Table 2. Some part of leaves turned yellowish by this time on the medium without any hormone. Multiple shoots were induced on the medium containing BAP, while the mean number of new shoots was reduced by the addition of NAA. Mean length of original shoots was longer on the medium containing BAP than on the medium without BAP. Rooting was observed on the medium without BAP irrespective of NAA concentration. Ratio of rooted shoot was increased to 80% by the concentration of 0.02 mg/l NAA.

Vitrificaion of shoot was observed on every medium containing hormones. Fifty to ninety percent of shoot was vitrificated on the medium containing NAA. Rate of vitrificated shoot was lower on the medium containing only BAP than on the medium containing NAA in combination. Although shoot elongation was longest on the medium containing 0.4 mg/l of BAP, 40% of shoots was vitrificated. On the medium supplemented with 0.2 mg/l BAP, shoots grew most healthy and rate of vitrificaion was the lowest.

Vitrification of tissue is a serious problem in micropropagation because it prevents regeneration of normal plants (Leshem *et al.*, 1988; Sato *et al.*, 1993). Vitrification is associated with high concentration of cytokinins (Ziv, 1991). In our study, shoots cultured on the medium with higher BAP concentration also showed vitrification more frequently. Since the selection of culturing on the medium with a lower BAP concentration is supposed to be effective to prevent vitrification, we selected 0.2 mg/l as an appropriate concentration of BAP for multiple shoot formation and healthy shoot growth.

This BAP concentration to 1/2 MS was used in plant regeneration from leaf protoplasts of *P. euphratica* and successful plant regeneration was attained (Kang *et al.*, 1996b). It means that 0.2 mg/l of BAP is a suitable concentration for shoot growth of this species in general.

After two weeks of culture, rooting was observed in all the media (Fig. 1). The highest ratio of rooting was achieved on the medium supplemented with 0.01 mg/l of NAA after three weeks of culture (Table 3). There are significant difference between the medium with NAA and that without NAA in the number of roots and the shoot growth. Kang and others used the medium without any hormone for the plant regeneration from leaf protoplast of *P.*

Table 3. Effect of NAA concentrations in 1/2MS on rooting of the shoot of *Populus euphratica**

NAA concentration (mg/l)	No. of shoot	Ratio of rooted shoot (%)	Mean no. of roots** (no.±S.E.)	Mean length of roots** (mm±S.E.)	Mean length of shoots** (mm±S.E.)
0	10	40	2.3±0.3	22.8±3.7	8.3±0.6
0.01	10	90	3.6±0.6	20.1±2.2	12.5±1.0
0.02	10	80	4.1±0.6	12.5±1.6	11.0±1.2
0.04	10	60	4.0±1.0	18.1±2.0	12.0±0.9

* Data was taken after 3 weeks of culture.
** Data was taken only from rooted explants.

Fig. 2. Plants of *Populus euphratica* in a green house. Plants had been established by *in vitro* culture and transplanted to the potting soils before 3 months. Plants were 50–80 cm tall.

euphratica (Kang *et al.*, 1996b). However, the ratio of rooted shoot was the lowest on the medium without NAA in our experiment. Then we selected 0.01 mg/l as an adequate NAA concentration because of good root development and shoot growth.

Almost all plantlets were successfully acclimatized by the procedure. The plantlets grew into 50–80 cm tall healthy stocks in a greenhouse after three month of transplanting (Fig. 2).

In this study, we showed a standard protocol for *in vitro* propagation of *P. euphratica*. It will serve not only for micropropagation but for various physiological researches also.

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Summary

A standard tissue culture system of *Populus euphratica* was established. Stem segments with axillary buds were excised from seedlings grown hydroponically in a greenhouse. They were surface-sterilized and cultured on MS or 1/2MS medium supplemented with various concentrations of BAP and NAA. Among the tested media, 1/2MS with 0.25 mg/l of BAP alone or in combination with 0.05 mg/l of NAA were optimal for shoot elongation and multiple shoot formation. After subculture on 1/2MS with 0.8 mg/l of BAP, the optimal BAP and NAA concentration was surveyed for rapid clonal propagation. Shoots grew most healthily and the rate of vitrification was the lowest on the 1/2MS medium supplemented with 0.2 mg/l of BAP. Rooting occurred on media without BAP irrespective of NAA concentration. The ratio of rooted shoots increased up to the highest of 90% at the concentration of 0.01 mg/l NAA after three weeks of culture. Almost all plantlets were successfully acclimatized and grew into 50–80 cm tall saplings in a greenhouse three months after transplanting to the potting soil.

Key words: *Populus euphratica*, tissue culture, plant regeneration, axillary buds, subculture

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Populus euphratica Oliv. の組織培養系の確立

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要　　旨

Populus euphratica の標準的な培養系を確立した。温室内における水耕栽培によって育てた *P. euphratica* の苗からえき芽のついた茎軸の小片を切り分け，表面殺菌後 BAP を添加した MS あるいは 1/2MS 培地に植え付けた。その結果，1/2MS に BAP を 0.25 mg/l 単独で，あるいは 0.05 mg/l の NAA とともに添加した培地が，シュートの伸長とマルチプルシュートの形成に最も適していた。0.8 mg/l の BAP を添加した 1/2MS 培地で一旦継代培養した後，継代培養に適した培地条件を検討した。その結果，0.2 mg/l の BAP を添加した培地でもっともガラス化するシュートの割合が少なく健全なシュートが得られた。BAP を含まない培地では NAA の添加の有無に関わらずシュートからの発根が認められた。NAA を 0.01 mg/l 添加した場合には発根率がもっとも高く 90% に達した。得られた植物体は，ほぼ 100% 順化が可能であり，温室内に置いて 3 ヶ月後には 50〜80 cm の苗木に育った。

キーワード: *Populus euphratica*, 組織培養, 植物体再生, えき芽, 継代培養

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A standard tissue culture system of *Populus euphratica* was established. Stem segments with axillary buds were surface-sterilized and cultured on MS or 1/2MS media supplemented with various concentrations of BAP. 1/2MS with 0.25 mg/*l* of BAP alone or in combination with 0.25 mg/*l* of NAA were optimal for primary culture. Shoots grew most healthily and the rate of vitrification was the lowest on the 1/2MS medium supplemented with 0.2 mg/*l* of BAP during subculture. The highest ratio of rooting was achieved on the medium supplemented with 0.01 mg/*l* of NAA after three weeks of culture. Plantlets were successfully acclimatized and grew in a greenhouse three months after transplanting to potting soil.

The Effect of Provenance Latitude on the Branching, Growth, and Phenological Characters for the Seedlings of *Larix* spp.

Hiromitsu KISANUKI and Akio KURAHASHI

To clarify the effect of provenance latitude on the growth and phenology of *Larix* spp., phenological observation and size measurement were made for 2-year-old seedlings of two species and four varieties, consisting of 17 strains, giving in total 277 individuals from the provenance between N63° and N36°. Branching differences were derived from the difference in response of lateral buds to day length. Seedling height was affected by the time of bud formation. There was a trend that the higher the provenance latitude, the earlier the time of bud formation, indicating that bud formation is related to the provenance latitude.