Some Effects of *Cryptomeria japonica* Inner Bark Extract on the Growth of *Pleurotus ostreatus*, and *Trichoderma harzianum*

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Introduction

The large quanties of bark stripped from trees harvested for lumber production have little economic value presently, but may prove to be a valuable resevoir of chemical products.[1] Climbing oil prices have stimulated the search for alternatives to products derived from fossil fuels.

Samejima et al[2] have been studying the chemistry of bark extractives and have observed that the rate of increase of the colony radius of *Trichoderma harzianum* Rifai aggr. was significantly reduced when grown on a semi-defined solid medium supplemented with 1.0—3.0% solutions of the hot water soluble fraction of the inner back of *Cryptomeria japonica* D. Don, Japanese cedar.

This study was undertaken to determine more about this inhibitory phenomenon, and to determine whether or not a selective fungicide existed that might have some value in the *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer, edible fungus industry.

Methods

This study was divided into three sets of experiments. In the first set *T. harzianum*, and *P. ostreatus* were grown on onion extract agar (OEA), and on OEA supplemented with a 1.0% solution of *C. japonica* inner bark extract (CJX; 1.0g per 100.0ml deionized water). OEA broth was made by chopping 250g of peeled onions, adding them to 1,000ml of deionized water, bringing the mixture to a boil, and letting it simmer gently for 30 minutes. The broth was poured through sterile cotton, and deionized water was used to restore the volume to 1,000ml. 25.0g glucose, 2.5g peptone, 0.15g KH_2PO_4, 0.10g $MgSO_4 . 7H_2O$, and 15.0 g of agar were added to the broth. The pH was adjusted to 5.5. The CJX was prepared by soaking 500g of *C. japonica* inner bark meal which had been passed through a 4.0mm square sieve in 2,000ml of deionized water at 60°C for 2 hours. This broth was concentrated using a rotary evaporator, and then freeze-dried. The OEA, and CJX supplemented OEA were poured into 1,000ml Erlenmeyer flasks stoppered with cotton, and covered with aluminum foil. They were sterilized at 15 psi, and 120°C for 15 minutes. Standard petri plates were

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filled axenically to a depth of approximately 7mm. Two types of inoculum: 5mm diameter plugs of OEA with either *P. ostreatus,* or *T. harzianum* hyphal tips were used. The plates were incubated at 26.5°C in a dark growth room, and daily increases in colony radius were recorded. This experiment was repeated three times, and there were 15 replicates of each treatment.

In the second experiment onion extract medium, and onion extract medium supplemented with a 1.0% solution of CJX were prepared as in the first experiment except that agar was omitted. Sterile medium (100ml) was used per treatment, and inoculated with 5mm plugs of OEA with either *P. ostresatus* or *T. harzianum* hyphal tips in 500ml Yamaguchi shake culture flasks. The flasks were fastened to a shaker, and incubated at 26.5°C in a dark growth room. After 3 and 13 days the fresh and dry weight of the mycelial pellets from each treatment were determined. This experiment was replicated three times, and there were five flasks per treatment per trial.

The third experiment involved a laboratory-scale facsimile of a Japanese commercial *P. ostreatus* production system. The control substrate was three parts of *C. japonica* sawdust, 12% moisture and passed through a 1.0mm square sieve, mixed uniformly with one part of rice bran, 8% moisture; the final moisture was adjusted to 66% of the total substrate mass. The test mixtures received either a 1.5%, 3.0%, or 4.5% CJX solution instead of deionized water when the final moisture content was corrected. To insure uniform substrate density 20.7g of each substrate was packed to a premarked line indicating a volume of 30.0ml in 20.0 by 2.0cm Pyrex test tubes. The tubes were stoppered with rolled paper plugs covered with aluminum foil and autoclaved for 30 minutes at 15 psi and 120°C on two consecutive days. The tubes were allowed to cool to room temperature, and were axenically inoculated with either *P. ostreatus* or *T. harzianum* hyphal tips. Incubaton was at 26.5°C in a dark growth room. Daily measurements were taken of the advance of mycelium through the substates. On the day that the mycelium was observed to have colonized all of the substrate, basidiomata induction or "kinkaki" was performed on tubes containing *P. ostreatus.* Kinkaki involves uncapping the tubes and removing the surface layer of aerial mycelium by scratching the substrate with a clean stainless steel spatula. The substrate was then covered in cold, approximately 18°C, water for 2 hours. The water was poured off and the uncapped tubes were transferred to a 12°C growth chamber maintained at 80—85% relative humidity. The substrate surfaces intercepted approximately 100 lux of continuous light from an incandescent source. The number of days to primordia formation were recorded. Also visual quantification of mycelial density of both *P. ostreatus* and *T. harzianum* was performed when the mycelium was observed to have colonized all of the substrate. Ratings were as follows: 0.0 indicates no growth through the substrate; 1.0 indicates low density growth throughout the substrate, but no area where the substrate's background colour was completely masked by the mycelium; 2. 0 indicated moderately dense growth throughout the substrate, where between one and five 1.0cm square areas of mycelium completely masked the substrate's background colour; 3. 0 indicated very dense mycelial growth where there were more than five 1.0cm square areas

of mycelium completely masking the substrate's background colour. At the same time *T. harzianum* sporulation on the surface of the substrate was visually quantified. The ratings were as follows: 0.0 indicates no detectable sporulation or a small ring of sporulation around the plug of inoculum not extending more than 2.0mm from the edge of the inoculum; 1.0 indicates up to one third of the substrate's surface area covered in sporulating myclium; 2.0 indicates between one third and two thirds of the subsrate's surface area covered in sporulating mycelium; and 3.0 indicates more than two thirds of the substrate's surface area covered in sporulating mycelium. There were three replicates of this experiment. In the first replicate there were five test tubes per treatment. In the second and third replicates there were ten test tubes per treatment.

In all three sets of experiments statistical analysis of variance was performed using an F-test at the 5% level of probability.

Results and Discussion

The results of the first set of experiments are presented in Figures 1 and 2, colony diameter verses time of *P. ostreatus* and *T. harzianum* on the control and test medium. Figure 1 indicates that neither the rate of colony diameter increase nor the lag before growth of *P. ostreatus* is detected, differ significantly between the control and the test medium.

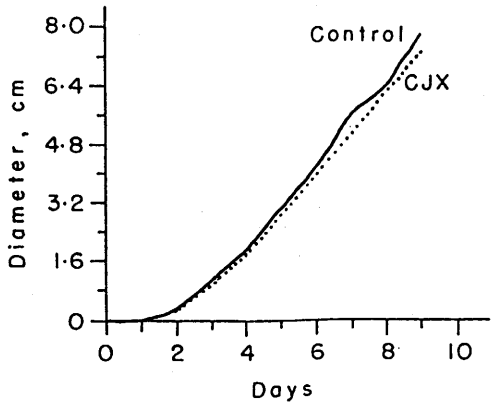

Fig. 1 *P. ostreatus* colony diameter versus time.

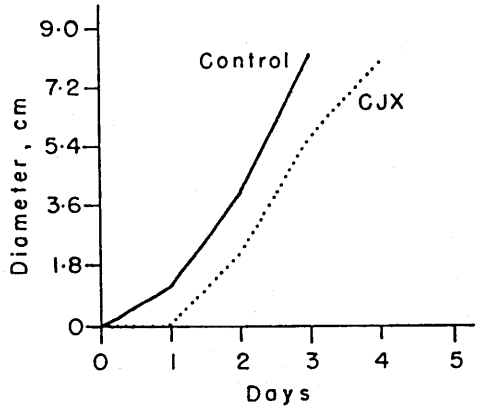

Fig. 2 *T. harzianum* colony diameter versus time.

Figure 2 indicates that *T. harzianum* hyphal tip inoculum is significantly delayed by approximately one day before growth is detected on the test medium compared to growth on the control. However, once growth is detected the slopes of the two lines are similar. These observations support earlier reports by Samejima et al[2] suggesting that CJX may be inhibitory to *T. harzianum,* but not *P. ostreatus.*

The results of the second set of experiments evaluating the rate of increase of mycelial mass production are summarized in Table 1. The dry weight results indicate significant differences in the rate of increase of mycelial mass in both test organisms. After 3 days the dry weight of *P. ostreatus* is undetectable after being cultured in the test medium, but 0.087g in the control medium. After 13 days the mean dry weight of the *P. ostreatus* mycelial pellet from the control was significantly heavier than the test medium.After 3 days the mean dry weight of the *T. harzianum* mycelial pellet obtained from the test medium was 42.5% of the mean dry weight of the pellet obtained from the control medium. After 13 days the test treatment pellet was 60.7% of the control's mean dry weight.

Table 1 Mycelial Mass Production in Liquid Media

		after 3 days		after 13 days	
treatment	inoculum	d.w.*	s.d.**	d.w.*	s.d.**
control	*P. ostreatus*	.087	.002	.125	.049
CJX	*P. ostreatus*	.000	.000	.004	.004
control	*T. harzianum*	.315	.036	.774	.032
CJX	*T. harzianum*	.134	.023	.470	.112

*driy weight
**standard deviation

These results suggest that both organisms demonstrate a significantly lower rate of mycelial mass production in the presence of CJX. The *T. harzianum* results suggest that CJX may delay the start of the grand phase of mycelial growth which is consistent with the results of the first set of experiments.

The results of the third set of experiments are summarized in Figures 3, 4, 5, and Tables 2, and 3. Figure 3 indicates that detection of *P. ostreatus* mycelial growth on the control medium does not occur significantly sooner than on the test media containing 1.5% CJX. However, detection is significantly delayed on the media containing both 3.0%, and 4.5% CJX solutions. Also, the slopes of the lines of the control, the 1.5%, 3.0%, and 4.5% treatments are similar once growth is detected.

Figure 4 shows that when *T. harzianum* was grown on the control medium detection of mycelial growth occurred significantly later on the test media containing 3.0%, and 4.5 % CJX. No significant difference was observed in the time when growth was detected between the control and the medium containing 1.5% CJX. Also, the slopes of the lines of the control and of the test treatments are similar once growth is detected.

Table 2 suggests an inverse curvilinear relationship between the concentration of CJX in the medium solution and the amount of *T. harzianum* sporulation. The type of quantification employed in this experiment does not permit a statistical correlation to be determined.

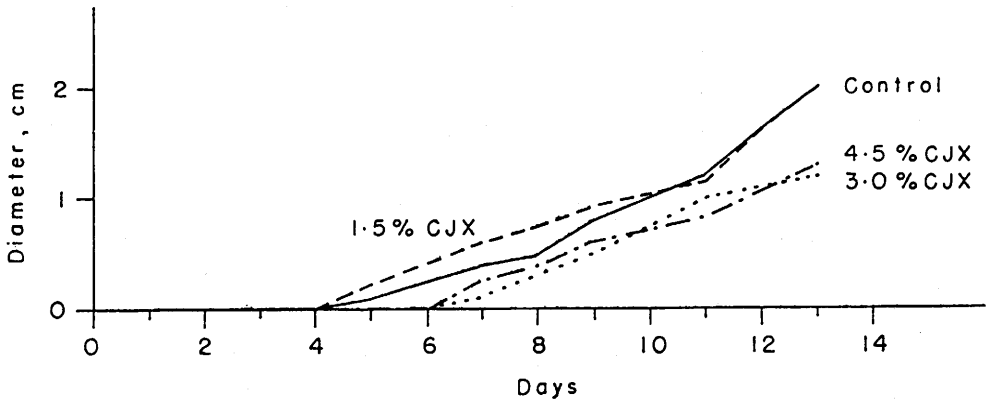

Fig. 3 Days for *P. ostreatus* to colonize sawdust substrates

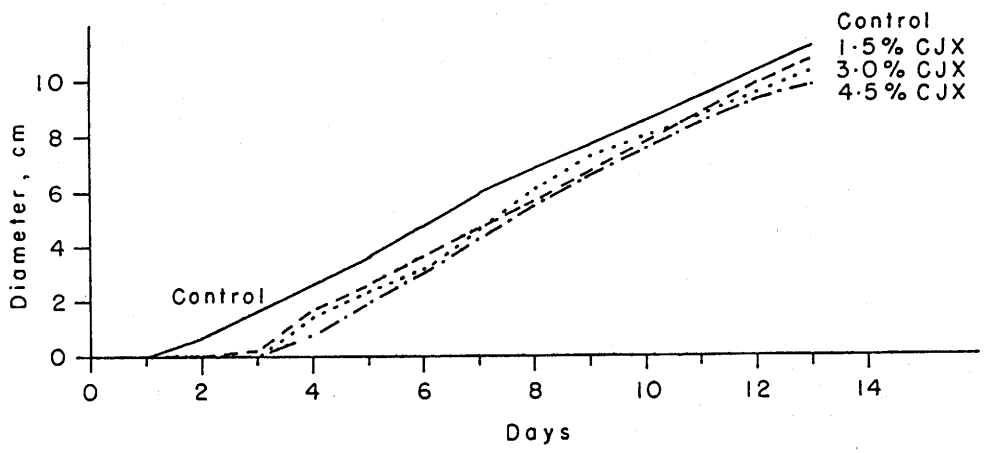

Fig. 4 Days for *T. harzianum* to colonize sawdust substrates.

Table 2 Visual Quantification of *T. harzianum* Sporulation

	mean rating	s.d.*
control	3.0	0.0
1.5% CJX	2.8	0.4
3.0% CJX	1.0	0.0
4.5% CJX	1.0	0.0

*standard deviation

Table 3 also suggests an inverse curvilinear relationship between the concentration of CJX in the medium solution and the mycelial density of both *T. harzianum* and *P. ostreatus*. Again, this method of quantification does not permit a statistical correlation.

Figure 5 is a histogram showing the mean number of days of both vegetative growth and the days to the observation of *P. ostreatus* primordia on the four media. A progressive increase in the mean number of days of vegetative growth in proportion to the increasing concentration of CJX is demonstrated, although a significant difference was not detected between the control and the 1.5% CJX treatment. The period between kinkaki and the observation of primordia in the test media containing 1.5% and 3.0% CJX solutions do not differ significantly. However, no primordia were observed on 4.0% of the media containing a 4.5% CJX solution even after more than twice that the time required for primordia initiation on the control treatment had lapsed. When primordia were detected they developed significantly later than on the control. This experiment was not able to determine whether quantitative yield differences of basidiomata occurred when basidiomata primordia were initiated on media containing CJX.

Table 3 Visual Quantification of Mycelial Density

treatment	inoculum	mean rating	s.d.*
control	*P. ostreatus*	2.0	0.0
1.5% CJX	*P. ostreatus*	2.0	0.0
3.0% CJX	*P. ostreatus*	2.0	0.0
4.5% CJX	*P. ostreatus*	1.6	0.5
control	*T. harzianum*	3.0	0.0
1.5% CJX	*T. harzianum*	2.8	0.4
3.0% CJX	*T. harzianum*	2.2	0.4
4.5% CJX	*T. harzianum*	1.4	0.5

*standard deviation

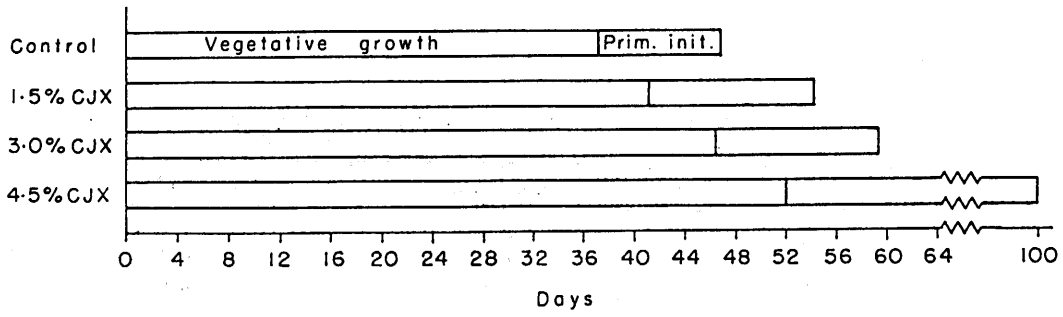

Fig. 5 Days for *P. ostreatus* to colonize sawdust substrates vegetatively and then to initiate primordia.

Summary

1. The hot water soluble fraction of the inner back of *C. japonica* significantly inhibits the growth of *T. harzianum* when growth is evaluated as either the rate of increase of colony diameter on a semi-defined solid medium, or the rate of mycelial mass production in a semi-defined liquid medium.
2. The mycelial of *T. harzianum* appears to decrease in proportion to increasing concentrations of the hot water soluble fraction of the inner back of *C. japonica* when *T. harzianum* is grown on a commercial substrate used for *P. ostreatus*.
3. Also, the amount of *T. harzianum* sporulation appears to decrease in proportion to increasing concentrations of CJX when *T. harzianum* is grown on a commercial substrate used for *P. ostreatus* production.
4. The hot water soluble fraction of the inner back of *C. japonica* significantly increases the time required for *P. ostreatus* to colonize a commercial substrate used for *P. ostreatus* production when the concentration of CJX is or exceeds 3.0% of the substrate solution.
5. Also, the time required for *P. ostreatus* to differentiate basidiomata primordia increases with the concentration of CJX present in a commercial substrate used for *P. ostreatus* production.

The advantage of incorporating a 1.0% solution of CJX into commercial *P. ostreatus* medium would be both to delay the development of *T. harzianum* inoculum present in the medium, and to reduce the production of spores in *P. ostreatus* production areas. However, supplementation of more than a 3.0% CJX solution has been shown to delay detrimentally the time of *P. ostreatus* primordia initiation.

The use of CJX in *P. ostreatus* production may be limited, but other applications should be considered because fungal growth inhibitory properties were demonstrated.

References

1) T. Yoshimoto et al: Mokuzai Gakkaishi, **30**, 335-339 (1984)
2) M. Samejima and T. Yoshimoto: ibid, **30**, 413-416 (1984)

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スギ内樹皮抽出物のヒラタケとトリコデルマ生育への効果

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要　　旨

スギ内樹皮の熱水抽出物がヒラタケ *Pleurotus ostreatus* 及びその害菌トリコデルマ *Trichpderma harzianum* に与える効果を追究し，次の事柄を明らかにした。

(1) 寒天培地，液体培地では抽出物は両菌の生育を妨げた。前者ではトリコデルマの生育半径について，後者ではヒラタケの菌体量について顕著に抽出物阻害効果が現われた。

(2) ヒラタケ生産に使われている木粉・米ぬか培地では抽出物の阻害効果はトリコデルマで顕著にあらわれた。即ち抽出物の添加量を増すと，菌体量，小胞子化量が必ず減少した。ヒラタケでの菌生長量，原基発生所要日数も抽出物の作用をうけるが，低濃度抽出物存在下では，無添加の場合とくらべて著しくは変らなかった。

(3) スギ内樹皮抽出物を１％程度含む水でヒラタケ生産用木粉・米ぬか培地を作ると，そこでのヒラタケの胞子形成は若干妨げられ，トリコデルマ菌糸の生長は遅れた。