Induction of Phenolic Compounds in Wheat (*Triticum aestivum* L.) Tissue Cultures by Streptomycin

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The tissue cultures of wheat (*Triticum aestivum* L.) were induced from the mature embryos (explants) of the dry grains and grown on MS medium containing kinetin (0.1 mg/l) and 2,4 D (1.0 mg/l). The cultures were incubated for two weeks at (25 ± 2) °C under a light/dark regime (16 h light daily). The formed calli were subcultured at the beginning of the stationary growth phase (15 days) with fresh MS medium containing 0, 5, 10, 25, 50, 100, 150 mg/l streptomycin elicitor and maintained for two weeks for three subcultures. A significant increase in phenylalanine ammonia lyase (PAL) activity coincided with the increase of the total phenolic compounds after elicitation with streptomycin. Maximum induction was recorded during the first two weeks, then gradually declined during the rest of the experimental period, but the values attained were still markedly higher than that of the control. The endogenous cinnamic acid content was also increased significantly with the increase in PAL activity making about 2–18% of the total phenolic acids. The growth and accumulation of phenolic compounds were inversely related. However, accumulation of phenolic compounds became limited for growth of wheat tissue culture especially during the long term cultivation.

Key words: Wheat (*Triticum aestivum*), Tissue Culture, Phenolic Compounds

Introduction

In vitro plant cell cultivation is a recent development, and the application of plant cell products of high yield of these compounds holds great promise. The objectives of many recent industries are to develop plant tissue culture techniques to the stage where the plants yield secondary products more cheaply than extracting either the whole plant grown under natural conditions or synthesizing the products. Furthermore plant cell culture has been suggested as a route for the biosynthesis of secondary products, from plants difficult to grow in short supply, as a source of novel chemicals and as biotransformation systems.

All phenolics are derived from phenylalanine or tyrosine (which are formed by the shikimate pathway) and phenylalanine ammonia lyase (PAL) which catalyzes the deamination of phenylalanine yielding *trans*-cinnamic acid. The formation of phenylpropanoid phytoalexins after pathogen infection involves a very rapid induction of PAL (DeAscensao and Dubery, 2000; Vâsquez *et al*., 2004). The aim of this study was to induce the synthesis of phenolic compounds and associated PAL activity by the aminoglycoside antibiotic streptomycin (elicitor) in wheat tissue cultures.

Materials and Methods

Establishment and growth of tissue cultures

Mature embryos (explants) were taken from the dry grains of wheat (*Triticum aestivum* L. cv. Gemmiza 1). The grains were first surface-sterilized by immersing in 70% ethanol for 1 min followed by 0.1% mercury chloride for 15 min, then washed with and soaked in sterile distilled water for 24 h before the embryos were aseptically exised. These were placed in 9-cm Petri dishes containing 20 ml of sterilized MS agar medium (Murashige and Skoog, 1962) containing kinetin (0.1 mg/l) and 2,4 D (1.0 mg/l). The pH value of the media was adjusted to 5.8 before addition of the agar. Each dish contained 5 embryos. The cultures were incubated for 2 weeks at (25 ± 2) °C under a light/dark regime (16 h light daily). The formed calli were transferred at the beginning of the stationary growth phase (15 d) to fresh MS medium containing 0, 5, 10, 25, 50, 100, 150 mg/l streptomycin and maintained for 2 weeks on the respective treatment for three subcultures of two-weeks interval. The fresh weight, total phenolic compounds and PAL activity of the callus tissues were recorded for each treatment.

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Extraction and assay of PAL (EC 4.3.1.5)

Extraction of the fresh callus was carried out by phosphate buffer, pH 7.0, at 4 °C following the method of Lister *et al.* (1996). PAL activity was assayed by the modified method of Jangaard (1974). The amount of cinnamic acid produced in the assay mixture was determined by measuring the absorbance at 275 nm using a JENWAY 6305 UV/Vis spectrophotometer. The amount of enzyme catalyzing the formation of 1 μmol of cinnamic acid in 1 s at 40 °C was defined as one enzyme unit.

Extraction and quantification of total phenols

These were extracted from the callus tissue with hot water or methanol (Sumaryono *et al.*, 1991), then assayed quantitatively at A_{765} with the Folin-Ciocalteau reagent (Singleton and Rossi, 1965), and expressed as mg of caffeic acid per kg fresh weight (FW).

Cinnamic acid analysis

Phenolic acids were obtained from the methanolic extract of callus tissues ground in liquid nitrogen (Cvikrova *et al.*, 1988). Phenolic acids (especially cinnamic acid) were analyzed by means of reverse phase HPLC using a Shimadzu-SPO-6AV instrument with a C_{18} hypesil ODS column and two solvents: (A) 5% aqueous acetic acid and (B) acetonitrile. Elution conditions were as follows: flow rate 0.5 ml min^{-1}; linear gradient from 100% to 90% of A for 30 min, from 90% to 40% of A for 10 min, then from 40% to 25% of A for 10 min and finally from 25% to 0% of A (100% B) for 10 min. The column eluate was monitored at 275 nm using a UV detector. Authentic samples were used as references for quantitative analysis.

Determination of total protein

This was estimated by the method of Bradford (1976) with Coomassie Brilliant blue (G 250), using bovine serum albumin (BSA) as a standard.

Statistical analysis

The data shown are mean values ± SD. Differences between means were compared using the LSD at the 0.05 probability. Levels of significance are represented by different letters.

Results and Discussion

There was a significant decrease in the fresh weight of callus tissue by the increase of the streptomycin concentration. However, there was an insignificant increase with the 5 mg/l streptomycin concentration (Fig. 1). This inhibition of growth in response to streptomycin treatment may be due to inhibition of protein synthesis (Egorov, 1985).

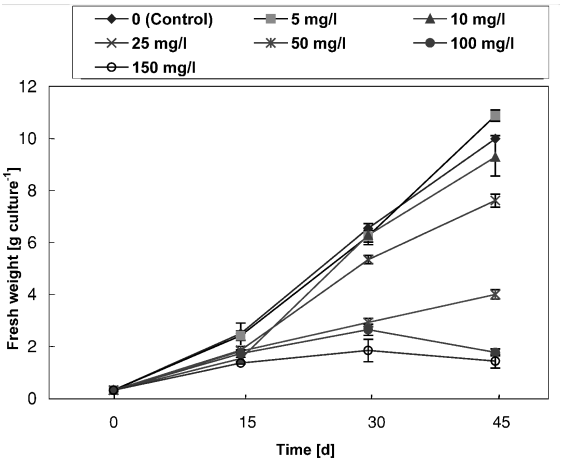

Fig. 1. Fresh weight of callus culture of wheat in response to treatment with streptomycin (0, 5, 10, 25, 50, 100, 150 mg/l). The calli in the stationary phase have been transferred to fresh medium (0 days of growth curve). Data are means ± SD, $n = 5–6$ replicates. There were statistically significant differences ($P < 0.05$) between fresh weight values of control and streptomycin-treated cultures except at 5 mg/l.

Luiz Antonio (1995) reported a reduction of callus formation and weight of avocado tissue culture by three antibiotics; nalidixic, chloramphenicol and streptomycin.

Addition of streptomycin to the culture media of wheat callus in the stationary phase inhibited the total protein content. Maximum inhibition was recorded at doses of 50 – 150 mg/l streptomycin (data not shown). At the end of the experiment the percentage of protein inhibition at 150 mg/l streptomycin was about 63. This inhibition of protein content of calli seems to be due to the inhibition of amino acid incorporation into protein at the final stages of the process without affecting the initial stage of activation of amino acids as previously reported by Egorov (1985).

The metabolism of phenolic compounds is regulated by the activity of various enzymes. The key step in biosynthesis of the phenylpropanoid skele-

ton in higher plants is the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia, a reaction catalyzed by PAL (Rösler *et al*., 1997).

The activity of PAL is often correlated with a change in the rate of accumulation of phenylpropanoids. This correlation suggests a possible casual relationship between the two biochemical events, and that PAL is the limiting factor in the biosynthetic pathways of such phenylpropanoids. PAL activity is affected by a number of factors including light, temperature, growth regulators, inhibitors of RNA and protein synthesis, wounding and mineral nutrition (Ruiz *et al*., 1998), and external stimuli such as microbial infections, UV radiation and chemical stress induce the synthesis of phenolic compounds (Daniel *et al*., 1999).

In this investigation addition of streptomycin elicitor to the callus culture of wheat in the stationary phase induced the activity of PAL which rose sharply reaching its maximum after the first 15 days then declined significantly during the rest of the experimental period, but the values attained were still markedly higher than that of the control (Fig. 2). Even at the 10 mg/l streptomycin concentration, the enzyme activity was approx. eight-fold the control value.

Under the prevailing conditions pronounced increase in PAL activity in wheat callus tissues coincided with the increase of total phenolic compounds (Fig. 3). Maximum induction was recorded during the first 15 days, then gradually declined. At the end of the experiment the total phenolics at 150 mg/l streptomycin were approx. seven-fold the control value. However, higher doses of the antibiotics caused extensive browning of the calli followed by cell lysis.

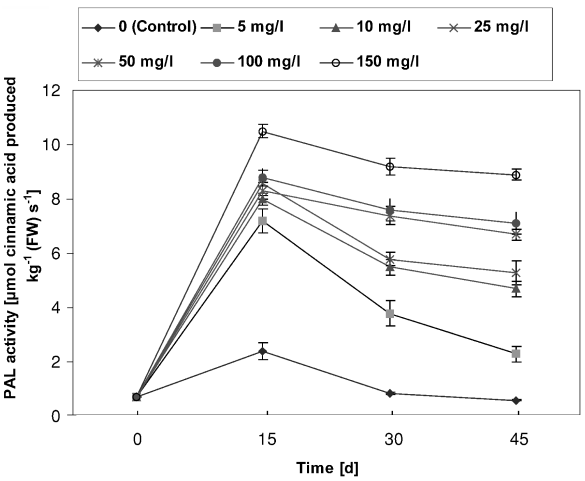

Fig. 2. Induction of PAL activity in callus tissues of wheat in response to treatments with streptomycin (0, 5, 10, 25, 50, 100, 150 mg/l). Data are means ± SD, $n = 3$. There were statistically significant differences ($P < 0.05$) between streptomycin-treated cultures and the control.

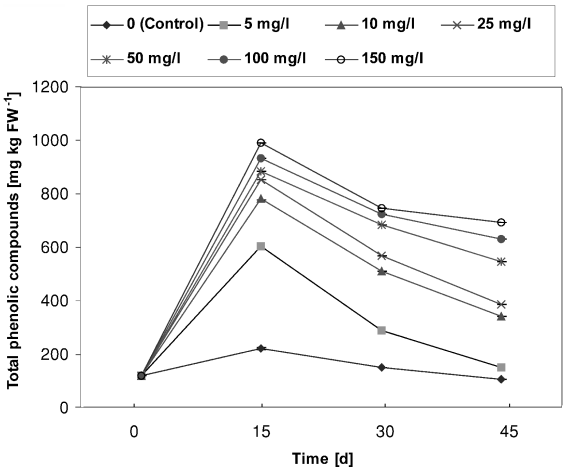

Fig. 3. Induction of total phenolic contents in callus tissue of wheat (methanolic extraction) in response to treatment with streptomycin (0, 5, 10, 25, 50, 100, 150 mg/l). Data are means ± SD, $n = 3$. There were statistically significant differences ($P < 0.05$) between streptomycin-treated cultures and the control.

Addition of yeast extract to the growth medium of Java tea suspension culture stimulated rosmarinic acid up to ten-fold of the control and PAL activity showed about 20-fold increase (Sumaryono and Proksch, 1993). Also, Vâsquez *et al*. (2004) reported that phenylpropanoids and PAL activity were induced by a yeast elicitor in cassava suspension cultures and leaves. Moreover, a significant increase in phenolic compounds in *Hypericum perforatum* cell suspension cultures after elicitation with *Colletotrichum gloeosporioides* was reported by Conceicâo *et al*. (2006).

Mancinelli *et al*. (1976) reported that streptomycin and chloramphenicol inhibited the development of the photosynthetic apparatus and enhanced anthocyanin in tomato and red cabbage seedlings. Cvikrova *et al*. (1994) reported that the sharp increase in PAL activity in tobacco and alfalfa cell suspension cultures was accompanied by an elevated accumulation of phenolic acids.

In the present investigation, the content of endogenous cinnamic acid, the product of phenylala-

nine deamination, was estimated at the end of the experiment (Table I); it increased significantly with the increase in PAL activity (together with the phenolic compounds) making about 2–18% of the total phenolic acid content in wheat tissue culture.

Hakulinen *et al*. (1999) reported that salicin, chlorogenic acid and cinnamic acid derivatives were significantly higher in rust-infected plants than in the controls. Also, Cvikrova *et al*. (1999) reported that the inhibition of phenylpropanoid biosynthesis in *Medicago sativa* suspension culture by AIP (2-aminoindan-2-phosphoric acid), a potent inhibitor of PAL, resulted in a marked reduction in the amount of hydroxy-cinnamic acid derivatives within a few hours after inoculation.

McKeehen (1999) suggested that accumulation of *p*-coumaric and ferulic acids in grains of six wheat cultivars at all stages of development appears to be related to *Fusarium* resistance.

The present study suggests that growth and accumulation of phenolic compounds are inversely related. The accumulation of phenolic compounds becomes limiting for growth especially during long term cultivation (Fig. 1). Similar results were previously obtained by Vaughn and Duke (1984). Smith-Becker *et al*. (1998) reported that the activities of polyphenol oxidase, peroxidase and PAL were increased in plants treated with various abiotic and biotic inducers. However, Cvikrova *et al*. (1999) concluded that the inhibition of phenylpropanoid metabolism stimulated cell division during the growth cycle of alfalfa suspension cultures.

Table I. Induction of cinnamic acid in callus tissue of wheat in response to treatment with streptomycin after 45 d. Data are means ± SD, $n = 3$. Values carrying different letters are significantly different at $P < 0.05$ corresponding to the control.

Treatment	Cinnamic acid [μmol/kg^{-1} FW]	Cinnamic acid as % of total phenolic acids
0.0 (control)	0.35 ± 0.03^{f}	2.40 ± 0.11^{g}
5	1.96 ± 0.083^{e}	3.31 ± 0.21^{f}
10	2.63 ± 0.18^{d}	7.88 ± 0.08^{e}
25	2.83 ± 0.16^{cd}	11.27 ± 0.16^{d}
50	3.02 ± 0.12^{c}	14.98 ± 0.11^{c}
100	3.37 ± 0.02^{b}	16.71 ± 0.19^{b}
150	6.64 ± 0.18^{a}	18.41 ± 0.31^{a}

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