Phenolic Content in Differentiated Tissue Cultures of Untransformed and *Agrobacterium*-Transformed Roots of Anise (*Pimpinella anisum* L.)

Nuri Andarwulan[†,‡] and Kalidas Shetty*[,†]

Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, and Department of Food Technology and Human Nutrition, Bogor Agricultural University, Indonesia

To investigate the role of differentiation of anise tissue cultures on total phenolic and anethole contents, benzylaminopurine- and thidiazuron-induced shoot cultures were generated from roots of the A-8 clonal line and its *Agrobacterium rhizogenes*-induced genetically transformed derivative JB-10. Embryogenic cultures were induced following 2,4-D treatment. Root cultures were multiplied on hormone-free medium. The effect of proline on differentiation and phenolic synthesis was also investigated. GC/MS studies indicate that anethole was not produced in root or other differentiated cultures. The predominant phenolic metabolite, however, was an anethole precursor, epoxypseudoisoeugenol-2-methylbutyrate (EPB). Total phenolics and EPB contents were highest in root cultures, which also correlated with higher proline content. Embryo and shoot cultures had reduced phenolic level and EPB and proline contents. Antioxidant activity in all differentiating cultures was high on day 60 compared to that on day 30, and there was no significant difference between differentiating tissues. This indicated that antioxidant protection might be linked not only to phenolics but to other nonphenolic metabolites as well.

Keywords: *Differentiated tissue cultures; untransformed roots; Agrobacterium-transformed roots; Pimpinella anisum; phenolics; free proline; epoxypseudoisoeugenol-2-methylbutyrate (EPB)*

INTRODUCTION

Dietary phenolic ingredients from plants are being investigated for their antimicrobial and antioxidant properties for uses such as natural food preservatives and disease prevention. *Pimpinella anisum* (anise) is an Umbelliferous plant, the leaf oil of which contains phenylpropanoids including *trans*-anethole, *trans*-pseudoisoeugenol-2-methylbutyrate, and myristicin, together with terpenoids such as β-bisabolene (Reichling et al., 1985). Eight phenylpropenyl esters were identified in extract of seed from six *Pimpinella* species. Six of these, including an anethole precursor, epoxypseudoisoeugenol-2-methylbutyrate (EPB), were successfully isolated and tested for antigermination activity (Kleiman et al., 1988). The alkylbenzene, anethole, derived from anise seeds exhibits antimicrobial activity (Himejima and Kubo, 1993; Curtis et al., 1996) and antimutagenic activity (Rompelberg et al., 1993). Because anise is a highly cross-pollinating species, the level of anethole and EPB produced in heterogeneous seeds is highly variable. To overcome the problem, production of anethole in undifferentiated callus cultures for potential bioreactor production has proved to be a failure. Therefore, we are exploring the use of differentiated root, shoot, and embryo cultures for this purpose.

Shoot-based organ cultures of anise have been reported to accumulate anethole, pseudoisoeugenol-2-methylbutyrate, and epoxypseudoisoeugenol-2-methylbutyrate, whereas root-based organ cultures accumulated pseudoisoeugenol-2-methylbutyrate only (Reichling et al., 1988). Other research has found that the essential oil of the transformed and untransformed shoot cultures of anise with *Agrobacterium tumefaciens* contains β-bisabolene, *trans*-pseudoisoeugenol-2-methylbutyrate, and *trans*-anethole (Salem and Charlwood, 1995). The latter work found that the leaf- and root-differentiating callus culture of *Pimpinella major* promoted the accumulation of epoxypseudoisoeugenol tiglate (Merkel and Reichling, 1990).

Transformed root-based organ cultures for secondary metabolite production can be obtained following infection of many dicotyledonous plant tissues with *Agrobacterium rhizogenes* [see review by Hamill and Rhodes (1993); Pellegrineschi et al., 1994; Paniego and Giuiletti, 1996]. The production of transformed roots through the use of *A. rhizogenes* vectors may also permit the targeted transfer of genes, which code for specific enzymes of the biosynthetic pathway. We have isolated clonal anise roots that were untransformed from which *A. rhizogenes*-transformed roots were also generated.

Considering the generally enhanced biosynthetic capacities of transformed anise roots cultures compared with that of their untransformed counterparts [see review by Hamill and Rhodes (1993); Pellegrineschi et al., 1994; Paniego and Giuiletti, 1996], it would be interesting to investigate the role of differentiation on the contents of total phenolic, anethole, and or other specific phenolic metabolites such as EPB. Furthermore, the successful development of *A. rhizogenes*-transformed

* Address correspondence to this author at the Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Box 31410, Amherst, MA 01003-1410 [telephone (413) 545-1022; fax (413) 545-1262; e-mail kalidas@foodsci.umass.edu].

† University of Massachusetts.

‡ Bogor Agricultural University.

10.1021/jf981214r CCC: $18.00 © 1999 American Chemical Society
Published on Web 03/10/1999

root cultures would be of considerable value as a model system to investigate the effects of gene transfer on the synthesis, transport, and storage of the lower phenylpropanoids and isoprenoids.

In this study, we investigated the role of differentiation of anise tissue cultures on total phenolic, anethole, and EPB contents. Furthermore, we also investigated the effect of proline on phenolic synthesis and antioxidant activity of the phenolic extract from differentiated tissues.

MATERIALS AND METHODS

Anise Root Clonal Lines. Two anise root clonal lines were used in this study: the A-8 clonal line and its genetically transformed derivative JB-10. The genetically transformed root clonal line JB-10 was isolated from the A-8 line following induction of rapidly proliferating thick hairy root mediated by *rol* gene transfer from *A. rhizogenes* (Bela and Shetty, 1996; Eguchi et al., 1997; Pellegrineschi et al., 1994).

Roots were grown on maintenance media for 30−40 days. Multiple roots were then subcultured and transferred to appropriate media for experiments. The subcultured anise roots of each line were stored at 20 °C in 24 h lighting for 30 and 60 days.

Induction of Differentiation of Anise Root Cultures. *Media Preparation.* Anise root explants were induced to produce multiple roots on Shenk and Hildebrandt−potassium (SHK) hormone-free medium (Shenk and Hildebrandt, 1972; Shetty and McKersie, 1993; Bela and Shetty, 1996) for maintenance. The SHK medium contains 3.2 g/L SH basal salt mixture (Sigma Chemical Co., St. Louis, MO), 10 mL/L Nitsch and Nitsch vitamin solution (Sigma Chemical Co.), 1.74 g/L K_2SO_4 (Fisher Scientific Co., Fair Lawn, NJ), 30 g/L sucrose (Domino Sugar Corp., New York, NY), 3 g/L Phytagel (Sigma Chemical Co.), and 1 L of distilled−deionized water. The pH of the solution was adjusted to 5.8 with 1 N NaOH or 1 N HCl. The solution was then sterilized in an autoclave at 120 °C for 20 min.

For experiments, both anise clonal lines in the SHK medium were transferred to a Murashige and Skoog (MS) salt medium (Murashige and Skoog, 1962) containing 4.5 μM 6-benzylaminopurine (BAP; Sigma Chemical Co.) or 0.5 μM 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea (thidiazuron/TDZ; Sigma Chemical Co.), where shoot cultures were induced (Figure 1a,b). Embryogenic cultures were induced from both lines following 4.4 μM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma Chemical Co.) treatment using SHK medium (Figure 1c). Root cultures from both lines were multiplied on MS hormone-free medium (Figure 1d).

The MS medium contains 4.33 g/L MS basal salt mixture (Sigma Chemical Co.), 1 mL/L Nitsch and Nitsch vitamin solution (Sigma Chemical Co.), 30 g/L sucrose (Domino Sugar Corp.), 3 g/L Phytagel (Sigma Chemical Co.), and 1 L of distilled−deionized water. The pH of the solution was adjusted to 5.8 with 1 N NaOH or 1 N HCl. The solution was then sterilized in an autoclave at 120 °C for 20 min.

Chemical Assays. (a) Total Phenolic Assay. Total phenolics were determined from a modified assay described by Chandler and Dodds, which was modified for our clonal lines using 50 mg of tissue (Shetty et al., 1995). Approximately 50 mg of tissue was placed in a small vial that contained 2.5 mL of 95% ethanol (ACS grade). After homogenization and centrifugation at 13000*g* for 10 min, 1 mL of supernatant was placed in a test tube to which 1 mL of 95% ethanol and 5 mL of filtered/deionized water were added. Folin−Ciocalteu reagent (50%, 0.5 mL; Sigma Chemical Co.) was added to each sample. After 5 min, 1 mL of 5% Na_2CO_3 (Fisher Scientific Co.) was added and mixed with a vortex mixer, and the reaction mixture was allowed to stand for 60 min in darkness. Samples were again homogenized with a vortex mixer, and absorbance was measured at 725 nm. A standard curve was prepared using gallic acid (Fisher Scientific Co.) in 95% ethanol.

(b) Free Proline Assay. Free proline in tissues was determined according to the method of Bates et al. (1973). Approximately 50 mg of fresh tissue was weighed and placed in 3 mL of 3% sulfosalicylic acid solution (Sigma Chemical Co.). After homogenization and centrifugation (13000*g*, 10 min) of the sample, 1 mL of supernatant was placed in a reaction test tube with marble as a cap. Glacial acetic acid (1 mL; Fisher Scientific Co.) and acid ninhydrin (1 mL; Sigma Chemical Co.; a mixture of 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) were added to each test tube, which was closed and heated in a 100 °C water bath for 1 h. After the sample had cooled in a cold room (5 °C) or in an ice bath for 15 min, 2 mL of ACS grade OmniSolv toluene (EM Science, Inc., Gibbstown, NJ) was added to every sample followed by vortexing for 20 s to mix thoroughly. The absorbance of the colored toluene phase (upper phase) was measured at 520 nm, and toluene was used as a blank. A standard curve was prepared using a series of proline (Sigma Chemical Co.) concentrations in 3% sulfosalicylic acid solution. Proline content was expressed as micromoles per gram of fresh weight (FW) of tissue.

(c) Antioxidant Activity Test. The antioxidant activity of the phenolic extract was evaluated using a β-carotene/linoleate model system described by Miller with modifications (Hammerschmidt and Pratt, 1978; Wanasundara et al., 1994). A solution of β-carotene (Sigma Chemical Co.) was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40 °C, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier (Aldrich Chemical Co., Milwaukee, WI), and 50 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots (5 mL) of this prepared emulsion were transferred into a series of tubes containing the equivalent of 2 mg of FW tissue (0.1 mL of phenolics extract). Reagent ethanol at 0.1 mL level was used as control. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 30 min by keeping the samples in a water bath at 50 °C. The protection factor used to express antioxidant activity was determined as the ratio of the sample's absorbance at 30 min to that of the control.

(d) GC Analysis for Potential Anethole Precursor Compound. Extracts of tissue cultures (∼50 mg) were obtained in 2.5 mL of ethanol. After homogenization, the samples were stored in stoppered vials at 4 °C for 2 weeks in the dark. Extracts were analyzed directly by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard model HP5890 series 2 chromatograph equipped with an SPB-5 fused silica capillary column (30 m $\times$ 0.25 mm i.d.; film thickness = 0.25 μm), the outlet of which was connected to a Hewlett-Packard HP5989A mass spectrometer. The GC with an oven temperature program was held at 80 °C for 1 min, increased to 290 °C at rate of 20 °C min^{-1}. The carrier gas was helium at a flow rate of 1.6 mL min^{-1}, and the sample size was 1 μL (injector temperature = 280 °C). The ion source temperature was 200 °C, and the ionizing potential was 70 eV with an accelerating voltage of 3 kV. For chemical ionization, ammonia was used at a pressure of 0.7 Torr. Quantitative analysis was carried out using GC-FID. The oven temperature was held at 50 °C for 1 min, increased to 240 °C at a rate of 10 °C/min, and held for a final time of 10 min. Calibration was done using *trans*-anethole as an internal standard.

RESULTS AND DISCUSSION

Total phenolics (Table 1) and anethole precursor (EPB) (Reichling and Martin, 1990) (Table 2) were highest in root cultures, which also correlated with higher proline content (Table 3). The long-range hypothesis of this research is that regulation of synthesis of phenolics is potentially coupled to proline synthesis (Figure 2). In this hypothesis NADPH-dependent proline synthesis increases due to stress and differentiation

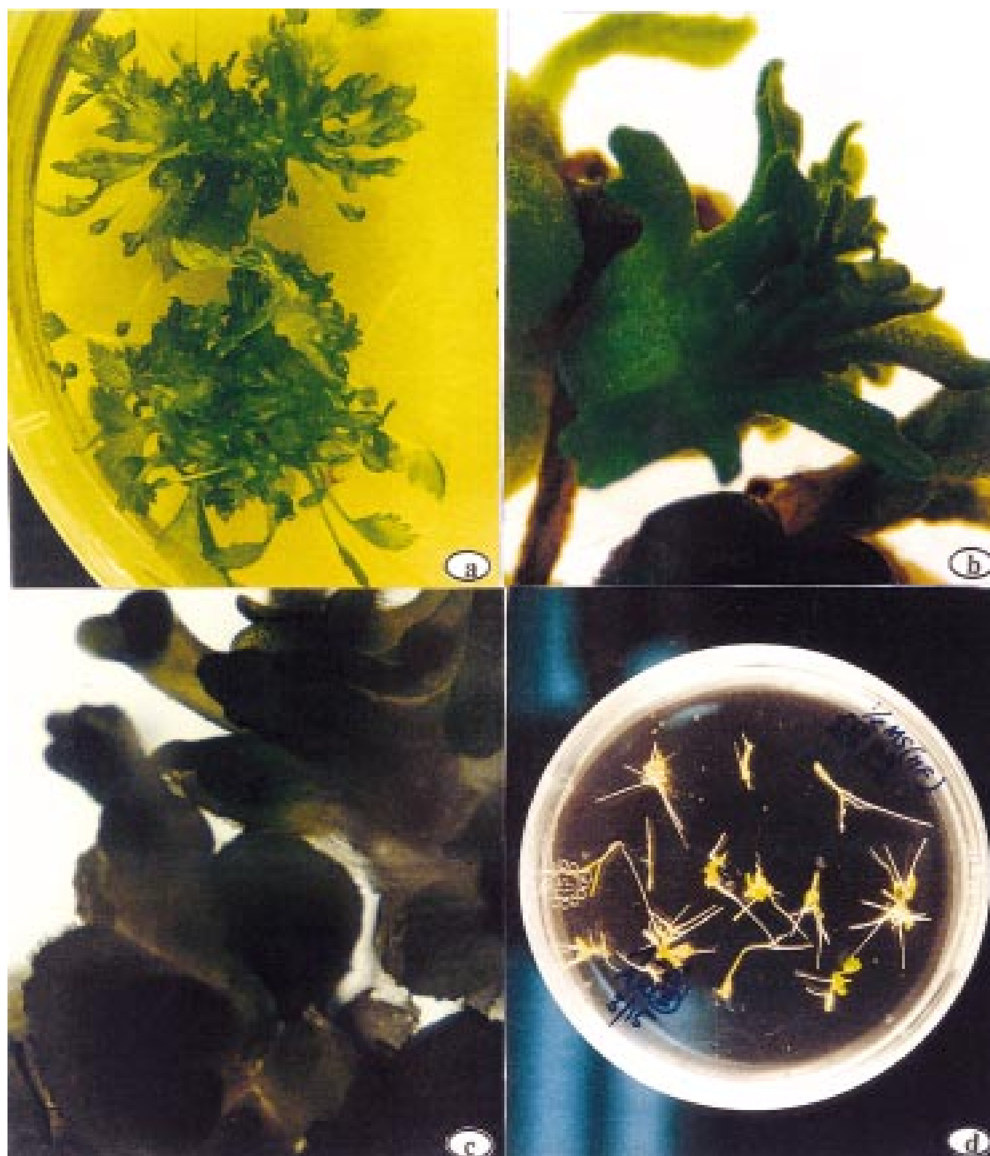

Figure 1. Anise tissue cultures: (a) shoot cultures were induced following BAP; (b) fused shoot cultures were induced following TDZ; (c) embryogenic cultures were induced following 2,4-D; (d) root cultures were multiplied on hormone-free medium.

Table 1. Concentration of Total Phenolics in Anise Tissue Cultures[a]

clonal line	hormone treatment	total phenolics (mg/g of fw) ± SD	
		day 30	day 60
A-8 (untransformed)	control	1.84 ± 0.15	1.64 ± 0.20
	TDZ	0.53 ± 0.04	0.39 ± 0.09
	BAP	0.80 ± 0.13	0.54 ± 0.07
	2,4-D	0.93 ± 0.16	0.98 ± 0.04
JB-10 (transformed)	control	2.21 ± 0.40	1.41 ± 0.27
	TDZ	0.53 ± 0.06	0.42 ± 0.05
	BAP	0.67 ± 0.09	0.45 ± 0.09
	2,4-D	0.65 ± 0.04	0.69 ± 0.14

[a] Each assay is a mean of four replicates.

Table 2. Concentration of the Anethole Precursor Compound EPB[a]

clonal line	hormone treatment	EPB (μg/g fw)	
		day 30	day 60
A-8 (untransformed)	control	1.28	1.14
	TDZ	0.40	nd[b]
	BAP	0.40	0.28
	2,4-D	0.19	0.52
JB-10 (transformed)	control	0.80	0.97
	TDZ	nd	nd
	BAP	0.24	nd
	2,4-D	nd	nd

[a] Each value is a mean of two independent assays. [b] nd, not detected.

responses, which in turn increases $NADP^+$ levels in the cytosol, causing $NADP^+$-induced glucose utilization to increase, thereby driving the pentose phosphate pathway toward the shikimate and phenylpropanoid pathway. This is based on the metabolic implication of differentiation, and stress-induced proline accumulation and oxidation in plants stimulate the proline synthesis-linked pentose phosphate pathway, thereby regulating the synthesis of ribose-5-phosphate and $NADPH_2$ toward purine synthesis and other anabolic pathways (Shetty, 1997; Hare and Cress, 1997).

Our results also showed that embryo and shoot cultures had reduced phenolic, anethole precursor (EPB) and proline contents. These results strengthened the hypothesis that during differentiation of root cultures, proline utilization via oxidation may be diverted for the synthesis of endogenous plant growth regulators such as indoleacetic acid (auxin) and isopentenyladenine

Table 3. Concentration of Free Proline in Anise Tissue Cultures[a]

clonal line	hormone treatment	free proline (μmol/g of fw) $\pm$ SD	
		day 30	day 60
A-8 (untransformed)	control	2.63 ± 0.44	0.94 ± 0.20
	TDZ	0.17 ± 0.05	0.16 ± 0.05
	BAP	0.21 ± 0.05	0.11 ± 0.01
	2,4-D	0.55 ± 0.12	0.20 ± 0.04
JB-10 (transformed)	control	1.33 ± 0.54	0.38 ± 0.15
	TDZ	0.61 ± 0.23	0.34 ± 0.10
	BAP	0.22 ± 0.11	0.33 ± 0.19
	2,4-D	0.56 ± 0.04	0.25 ± 0.13

[a] Each assay is a mean of four replicates.

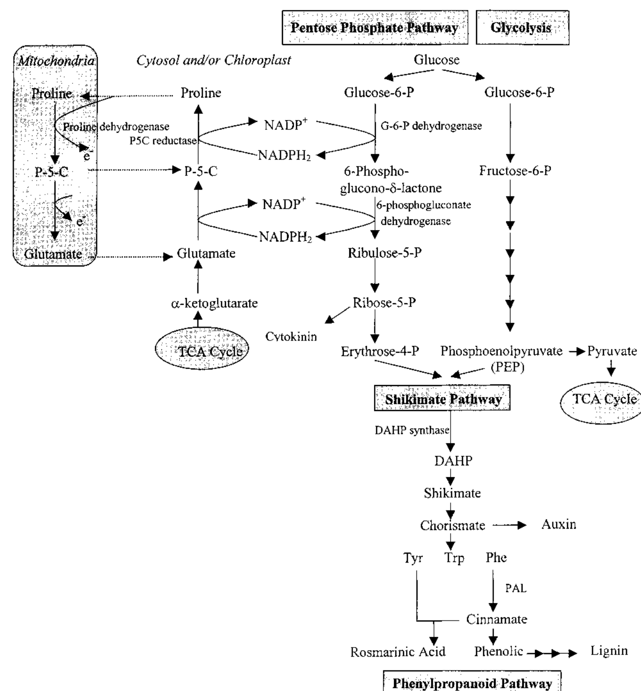

Figure 2. Proline-linked pentose phosphate pathway.

(cytokinin) needed for embryo and shoot formation, respectively, through the stimulation of the pentose phosphate pathway (Figure 2). There were no differences in total phenolics, free proline, and EPB in untransformed and transformed clonal lines. However, it is interesting to note from this experiment that TDZ treatment of transformed anise root cultures (line JB-10) produced higher free proline content than TDZ treatment of untransformed roots (line A-8) on day 30 (Table 2). This may stimulate endogenous auxin and cytokinin biosynthesis needed for tissue differentiation in response to TDZ. Additional studies to confirm the NADP/$NADPH_2$ ratios produced in response to proline turnover and production of the above anabolic products (phenolics, auxin, and cytokinin) will strongly support this hypothesis.

The concentrations of total phenolics, free proline, and anethole precursor (EPB) were higher at day 30 than at day 60 for both untransformed and transformed clonal lines. Another interesting result showed that the antioxidant activity in all differentiating cultures was high on day 60 compared to that on day 30. On either day, however, there was no significant difference in antioxidant activity between differentiating tissues (shoot, root, or somatic embryos) (Table 4). This indicated that antioxidant protection may be linked not only

Table 4. Antioxidant Activity of Phenolic Extract from Anise Tissue Cultures[a]

clonal line	hormone treatment	antioxidant activity (protection factor)	
		day 30	day 60
A-8 (untransformed)	control	1.52	1.63
	TDZ	1.43	1.87
	BAP	1.52	2.10
	2,4-D	1.47	2.17
JB-10 (transformed)	control	1.53	2.28
	TDZ	1.30	1.98
	BAP	1.40	2.01
	2,4-D	1.44	1.97

[a] Each assay is a mean of four replicates.

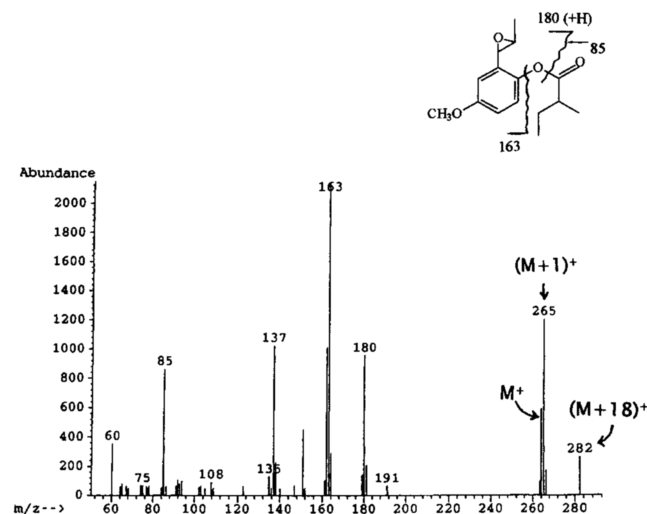

Figure 3. Chemical ionization mass spectrum of EPB.

to free phenolics but to other metabolites as well. It is clear from the formation of shoots and embryos that cytokinin and auxin contents were needed for differentiation of such tissue types. It is not clear at this time whether such purine and aromatic metabolites contribute to antioxidant activity as well.

GC/MS studies using chemical ionization technique showed that the anethole precursor EPB, with a molecular ion of 264, was confirmed (Figure 3). Studies have shown that anethole was a precursor of EPB (Reichling and Martin, 1990). EPB is known to regulate seed germination (Kleiman et al., 1988) and possibly vigor. Our anise root culture system could be targeted for large-scale production of EPB, which can be used for improving seed germination in food crops. Optimum seed germination and synchronization have been identified as critical for the release of phenolic metabolites in seeds. We have isolated clonal root cultures of anise that produce EPB at high levels, and its synthesis parallels high proline biosynthesis. This novel compound may in part contribute to the high antioxidant activity of anise root and differentiating cultures and therefore could be targeted for potential food and nutraceutical applications.

CONCLUSION

In conclusion, anise root cultures are good sources of EPB. Total phenolic and EPB contents appear to be linked to proline content, thereby strengthening the hypothesis that it could be coupled to the pentose phosphate pathway. Further studies to measure the NADP/$NADPH_2$ ratios generated by the proline turn-

over will confirm the validity of this hypothesis. From an applied perspective, EPB, however, can be targeted for regulating seed germination and enhancement of phenolic antioxidants for emerging nutraceutical applications.

ACKNOWLEDGMENT

We give special thanks to Dr. Thomas L. Potter for excellent technical suggestions with the GC/MS studies.

LITERATURE CITED

Bates, L. S.; Waldren, R. P.; Teare, I. D. Rapid determination of free proline for water stress studies. *Plant Soil* **1973**, 39, 205–207.

Bela, J. S.; Shetty, K. *In vitro* developmental response of anise to growth regulators and establishment of a clonal propagation system. *Acta Horticult.* **1996**, *426*, 483–487.

Curtis, O. F.; Shetty, K.; Cassagnol, G.; Peleg, M. Comparison of the inhibitory and lethat effects of synthetic versions of plant metabolites (anethole, carvacrol, eugenol, and thymol) on a food spoilage yeast (*Debaromyces hansenii*). *Food Biotechnol.* **1996**, *10*, 55–73.

Eguchi, Y.; Bela, J. S.; Shetty, K. Simulation of somatic embryogenesis in anise (*Pimpinella anisum*) using fish protein hydrolysates. *J. Herbs, Spices Med. Plants* **1997**, *5*, 61–68.

Hamill, J. D.; Rhodes, M. J. Manipulating secondary metabolism in culture. In *Plant Biotechnology*; Grierson, D. Ed.; Lhagman & Hall: New York, 1993; Vol. 3, pp 179–209.

Hammerschmidt, P. A.; Pratt, D. E. Phenolic antioxidants of dried soybeans. *J. Food Sci.* **1978**, *43*, 556–559.

Hare, P. D.; Cress, W. A. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* **1997**, *21*, 79–102.

Himejima, M.; Kubo, I. Fungicidal activity of polygodial in combination with anethole and indole against *Candida albicans*. *J. Agric. Food Chem*. **1993**, *41*, 1776–1779.

Kleiman, R.; Plattner, R. D.; Weisleder, D. Antigermination activity of phenylpropenoids from the genus *Pimpinella*. *J. Nat. Prod*. **1988**, *51*, 249–256.

Merkel, B.; Reichling, J. Comparative investigation on formation and accumulation of rare phenylpropenoids in plants and *in vitro* cultures of *Pimpinella major. Z. Naturforsch.* **1990**, *45C*, 602–606.

Murashige, T.; Skoog, G. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497.

Paniego, N. B.; Giulietti, A. M. Artemisinin production by *Artemisia annua* L.-transformed organ cultures. *Enzyme Microb. Technol*. **1996**, *18*, 526–530.

Pellegrineschi, A.; Damon, J.-P.; Valtorta, M.; Paillard, N.; Tepfer, D. Improvement of ornamental characters and fragrance production in lemon-scented geranium through genetic transformation by *Agrobacterium rhizogenes*. *Bio/Technology* **1994**, *12*, 64–68.

Reichling, J.; Martin, R. Further studies on the biosynthesis on the pseudoisoeugenols in tissue cultures of *Pimpinella anisum* L. *Z. Naturforsch*. **1990**, *45C*, 942–948.

Reichling, J.; Becker, H.; Martin, H.; Burkhardt, G. Comparative studies on the production and accumulation of essential oil in the whole plant and in the cell culture of *Pimpinella anisum* L. *Z. Naturforsch*. **1985**, *40C*, 465–468.

Reichling, J.; Martin, R.; Thron, U. Production and accumulation of phenylpropenoids in tissue and organ cultures of *Pimpinella anisum* L. *Z. Naturforsch*. **1988**, *43C*, 42–46.

Rompelberg, C. J. M.; Verhagen, H.; Van Bladeren, P. J. Effects of the naturally occurring alkylbenzenes eugenol and trans-anethole on drug-metabolizing enzymes in the rat liver. *Food Chem. Toxicol*. **1993**, *31*, 637–645.

Salem, K. M. S. A.; Charwood, B. V. Accumulation of essential oils by *Agrobacterium tumefaciens*-transformed shoot cultures of *Pimpinella anisum*. *Plant Cell, Tissue Organ Cult.* **1995**, *40*, 209–215.

Shenk, R. V.; Hildebrandt, A. C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot*. **1972**, *50*, 199–204.

Shetty, K. Biotechnology to harness the benefits of dietary phenolics; focus on Lamiaceae. *Asia Pacific J. Clin. Nutr*. **1997**, *63*, 162–171.

Shetty, K.; McKersie. Proline, thioproline and potassium mediated stimulation of somatic embryogenesis in alfalfa (*Medicago sativa* L.). *Plant Sci.* **1993**, *88*, 185–193.

Shetty, K.; Curtis, O. F.; Levin, R. E.; Witkowsky, R.; Ang, W. Prevention of vitrification associated with in vitro shoot culture of oregano (*Origanum vulgare*) by *Pseudomonas* spp. *J. Plant Physiol*. **1995**, *147*, 447–451.

Wanasundara, U.; Amarowicz, R.; Shahidi, F. Isolation and identification of an antioxidative component in canola meal. *J. Agric. Food Chem*. **1994**, *42*, 1285–1290.

Received for review November 9, 1998. Revised manuscript received January 26, 1999. Accepted January 27, 1999. We thank the Indonesian government (URGE) exchange program and the Indonesian Cultural Foundation for a grant supporting N.A.

JF981214R