Stimulation of Benzyladenine-Induced in Vitro Shoot Organogenesis and Endogenous Proline in Melon (*Cucumis melo* L.) by Fish Protein Hydrolysates in Combination with Proline Analogues

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A previous study demonstrated that proline is beneficial for improving melon in vitro shoot organogenesis. A natural source of proline and proline precursors can be obtained from fish protein hydrolysates (FPH), a byproduct of the fishery industry. Proline analogues azetidine-2-carboxylate and hydroxyproline in combination with standardized FPH were used to stimulate proline synthesis and benzyladenine-induced shoot organogenesis by exploiting the proposed proline-linked pentose phosphate pathway (PPP). In the presence of elevated levels of endogenous proline, potential stimulation of cytokinins and auxin may occur via the PPP and shikimate pathways, respectively. Treatments with FPH singly and in combination with the above proline analogues significantly increased the endogenous proline content and the extent of differentiation, suggesting that in vitro organogenesis is closely linked to proline synthesis, strengthening the hypothesis that purine metabolism via the proline-linked PPP may be important for organogenesis. Thioproline addition resulted in increased proline levels but without corresponding stimulation of organogenesis. This study also provides potential use of fishery waste for value-added application in plant micropropagation industry.

Keywords: *Azetidine-2-carboxylate; benzyladenine; Cucumis melo; fish protein hydrolysate; hydroxyproline; pentose phosphate pathway; proline; shoot organogenesis; thioproline*

INTRODUCTION

Expression of novel proteins in transgenic melon (*Cucumis melo* L.), benefiting food and pharmaceutical industries, is being explored by our laboratory (Shetty et al., 1997). Additionally, melon provides an excellent in vitro system to study the role of proline and proline precursors in regulating benzyladenine-induced shoot organogenesis. Proline has been shown to stimulate plant somatic embryogenesis and benzyladenine (cytokinin)-induced shoot organogenesis (Armstrong and Green, 1985; Nuti Ronchi et al., 1984; Shetty et al., 1992; Kim et al., 1990, 1994; Milazzo et al., 1998).

Fish protein hydrolysates (FPH) are a common byproduct of the fishery industry and are produced in large quantities. They are a rich source of proline and proline precursors (Mackie, 1982) that have been demonstrated to be beneficial for improving in vitro tissue culture in plants (Eguchi et al., 1997, 1998). This potential for a natural source of proline, proline precursor (glutamate), and proline analogue [hydroxyproline (HP)] can be exploited for plant tissue culture and propagation and is being investigated by our laboratory.

In this study, we further investigated the effects of exogenous FPH singly and in combination with the proline analogues azetidine-2-carboxylate, HP, and thioproline (TP) on benzyladenine (BA)-induced in vitro shoot organogenesis in melon (*C. melo* L.) and the correlation between organogenesis and endogenous levels of proline. The role of the proposed proline-linked pentose phosphate pathway (PPP) in stimulating the synthesis of intermediary metabolites involved in purine and aromatic metabolism (Figure 1) is our proposed model for investigating these effects. In this model, the proposed proline-linked PPP drives ribose-5-phosphate toward purine synthesis and subsequently cytokinin and auxin biosynthesis in plant cells (Kohl et al., 1988; Milazzo et al., 1998). It has been demonstrated that purine synthesis in animal cells is based on such a model (Phang et al., 1982). In the proposed proline-linked PPP the interconversion of ribose-5-phosphate to phosphoribosyl pyrophosphate (PRPP) and to erythrose-4-phosphate may be critical for the biosynthesis of cytokinins and auxins (via the shikimate pathway), respectively, in addition to making $NADPH_2$ available for all anabolic reactions. These metabolic intermediaries, $NADPH_2$ and sugar phosphates, must be derived from the PPP (Schnarrenberger et al., 1995). An alternative oxidative phosphorylation step that utilizes proline as a reductant in place of NADH via the stimulation of the proline-linked redox cycle has been suggested (Phang, 1985; Rayapati and Stewart, 1991) and could be used to meet energy needs during organogenesis. The investigation of the proline-linked PPP in regulating the biosynthesis of cytokinins and auxins requires first the confirmation that the enhancement of benzyladenine-induced shoot organogenesis is correlated with concurrent enhancement of endogenous proline levels. Results reported here confirm this positive correlation between shoot organogenesis and endogenous proline levels in

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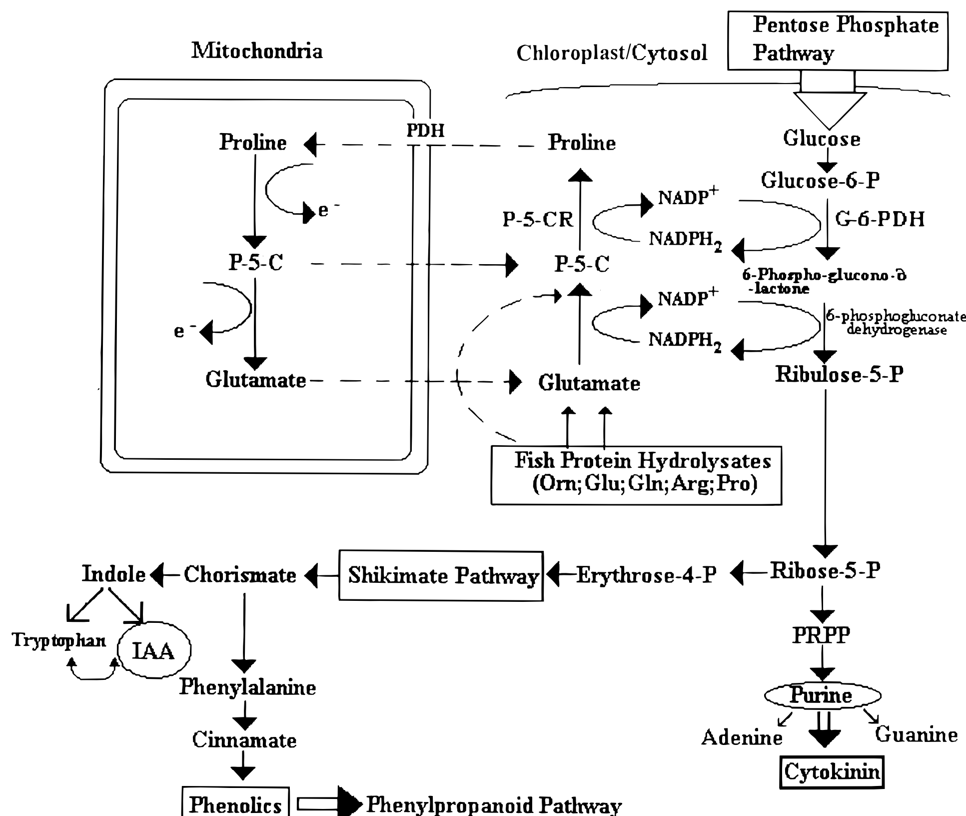

Figure 1. Proposed proline-linked PPP and its role in stimulation of BA-induced organogenesis in melon. Abbreviations: P5C, pyrroline-5-carboxylate; G6PDH, glucose-6-phosphate dehydrogenase; PDH, proline dehydrogenase; PRPP, phosphoribosyl pyrophosphate; IAA, indoleacetic acid.

response to FPH as well as provide a novel value-added application of FPH for plant in vitro propagation.

MATERIALS AND METHODS

Plant Material. Mature seeds of melon (*C. melo* L. var. Hales Best, NK Lawn and Garden Co., MN) were germinated for 48−72 h under 80 μmol m^{-2} s^{-1} at 25 °C on MS medium (Murashige and Skoog, 1962) with 1 mg/L benzyladenine (BA) and 3% (w/v) sucrose (pH 5.8) after removal of seed coat and disinfection. Disinfection was done by treating the seeds for 45 s in 70% (v/v) ethanol, followed by 25 min in 2% (v/v) sodium hypochlorite, and then rinsing by three serial washes in sterile distilled water to remove traces of the disinfectant. Following germination and cotyledon expansion for 48−72 h, sliced cotyledon explants were used to initiate shoot organogenesis (Shetty et al., 1992).

Cotyledon Explants and Initiation of Shoot Organogenesis. Expanded cotyledons were cut into 2 mm × 2 mm sections after the waxy cuticle layer was removed (Dirks and Van Buggenum, 1989). Twelve explants were obtained from each cotyledon (i.e., six explants from each cotyledon half). Individual explants were randomly placed on various shoot organogenesis solid media described below. Every treatment in this study had 36 explants for each of the individual replicates (i.e., nine explants/plate, four plates per individual treatment/replicate). Each Petri plate was 10 mm × 100 mm size and had 15 mL of medium. Samples were grown for 30−33 days at 25 °C and 40 μmol m^{-2} s^{-1} (18 h fluorescent light/6 h dark cycle). Experiments were repeated at least 10 times and results averaged.

Shoot Organogenesis Medium. All in vitro organogenesis media were based on MS medium with Nitsch and Nitsch vitamins (Nitsch and Nitsch, 1955; Shetty et al., 1995) containing 1 mg/L BA and 3% sucrose, with an adjusted pH of 5.8. Phytagel (Gellan gum; Sigma Chemical, St. Louis, MO) at 3 g/L was used as the solidifying agent. All treatment concentrations were adjusted to appropriate levels in the above

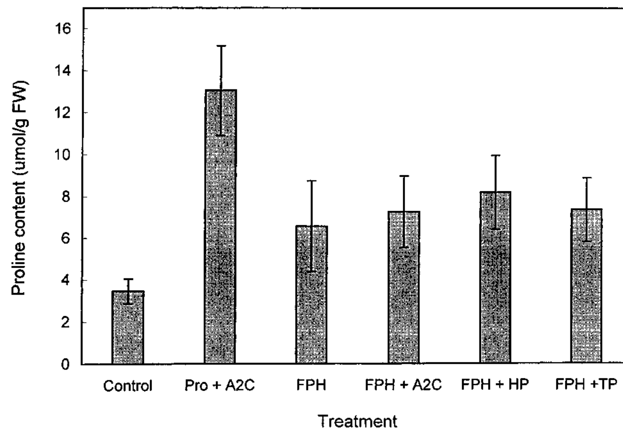

Figure 2. Endogenous proline content of multiple-shoot-forming cut cotyledons of melon following 30 days of treatment with FPH in combination with proline and proline analogues.

medium, and the mixture was sterilized by standard autoclaving. Concentrations of various supplements (all from Sigma) were as follows: proline (5 mM), azetidine-2-carboxylate (A2C) (0.05 mM), TP (0.5 mM), HP (0.5 mM), and concentrated standardized FPH (1 mL/L of medium containing >2.5 mM glutamic acid and proline) (FPH was from Conolley Sea Foods, Gloucester, MA). Treatment combinations are shown in Figure 2.

Proline Determination. The endogenous proline content in plant tissue was determined according to the method of Bates et al. (1973). About 50−99 mg of plant material (containing both callus and developing tissue) was blotted lightly on tissue paper, weighed, and placed in 2 mL of 3% (w/v) sulfosalicylic acid solution and homogenized. A total of 1.5 mL of the macerated sample was centrifuged at 13000*g* for 10 min in a microcentrifuge. One milliliter of supernatant was extracted and placed into a reaction tube to which 1.0 mL

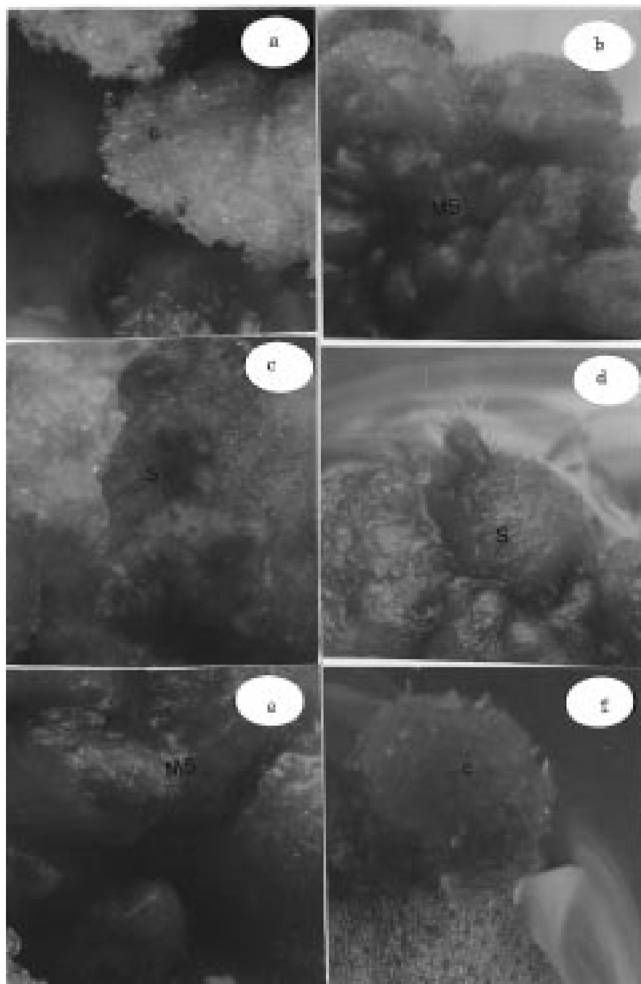

Figure 3. Multiple shoots induced on cut cotyledons after 30 days of treatment with the following materials: (a) control; (b) proline + A2C; (c) FPH; (d) FPH + A2C; (e) FPH + HP; (f) FPH + TP. (Magnification of 35×.; figure is reproduced here at 70% of the original.) Abbreviations: MS, multiple shoots; S, shoot; C, callus.

of glacial acetic acid and 1.0 mL of 2.5% (w/v) acid ninhydrin were added. Samples were heated in a 100 °C water bath for 1 h and then cooled for 15 min in an ice bath. A total of 2.0 mL of toluene was added and then vortexed for 20 s. The upper chromophore phase was measured at 520 nm using toluene as a blank. Standards were treated in the same manner as the samples and prepared simultaneously. Three samples per treatment were assayed per individual experiment. Proline is reported as micromoles per gram of fresh weight tissue. Experiments were repeated 10 times with similar trends, and the averages of 10 experiments are reported here. Proline content was measured on day 30 following the BA and various supplement treatments.

Organogenesis. Photographs of the individual treatment plates were taken using a stereomicroscope (Olympus SZ40, Tokyo, Japan) to show the extent of callus and/or multiple shoot formation after 30 days of growth under the conditions above, and a magnification of 35× was used (Figure 3).

RESULTS AND DISCUSSION

Endogenous proline levels increased significantly in cotyledons grown on MS−BA medium containing a combination of proline plus A2C, with concentrations of endogenous proline in these tissues almost 4 times that of the untreated control (Figure 2). Similarly, significantly higher levels of endogenous proline versus untreated control were obtained in tissue grown on MS−BA medium containing all treatments with FPH singly and FPH plus A2C, HP, and TP. Our previous study demonstrated that a high level of endogenous proline coupled with enhanced shoot organogenesis was obtained following treatment of melon explants with proline plus A2C (Milazzo et al., 1998). In comparing these results with treatments including FPH, the highest concentrations of endogenous proline were obtained from tissue grown on medium containing FPH plus HP (Figure 2), and this correlated with enhanced shoot organogenesis (Figure 3).

Reduced multiple shoot formation and excess callus tissue were observed when explants were grown on control MS medium containing standard inorganic sources of nitrogen with 1 mg/L benzyladenine. Stimulation of benzyladenine-induced in vitro shoot organogenesis directly from cut cotyledons was seen with treatments of FPH singly, FPH plus A2C, and FPH plus HP (Figure 3). Direct correlation of shoot organogenesis with increased levels of endogenous proline was observed in these cases. However, an increase in endogenous proline did not correlate with stimulation of shoot organogenesis in treatments with TP.

A2C is suggested to be a competitive inhibitor of proline dehydrogenase (PDH), an inner mitochondrial bound enzyme (Elthon and Stewart, 1984; Rayapati and Stewart, 1991). A2C is also known to inhibit differentiation of Leydig cells of fetal rat testis (Jost et al., 1988), and its effect can be reversed by treatments with exogenous proline. In our previous study, melon explants grown on MS medium with 1 mg/L BA and 0.05 mM A2C did not have endogenous proline concentrations significantly higher than control (Milazzo et al., 1998). In the presence of A2C, proline synthesis may be required to out-compete A2C, resulting in the immediate oxidation of proline to pyroline-5-carboxylate and glutamate and therefore resulting in lower proline accumulation. The PPP could be continuously stimulated as proline oxidation products may be recycled for proline synthesis, generating $NADPH_2$ and ribose-5-phosphate for purine synthesis (Phang, 1985). This proline redox cycle may operate more efficiently in the presence of FPH and A2C because of the potential deregulation of proline synthesis by A2C. Simultaneously, there were also high levels of exogenous proline provided by FPH for potentially initiating PDH-linked oxidation and maintenance of the proline-linked redox cycle; therefore, stimulation of the PPP may be maintained continuously. The proline-linked redox cycle required for differentiation may be maintained by PDH-mediated oxidation in the presence of FPH alone, without excessive synthesis as compared to proline plus A2C and FPH plus A2C.

HP is normally obtained during post-translational modification of certain plant cell wall proteins (Varner and Lin, 1989). In FPH extracts, HP is hydrolyzed from muscle tissues. Exogenous HP may compete with proline for incorporation into protein and cause aberrant proteins at the translational level. To outcompete HP, proline synthesis has to be stimulated. In our previous study, the addition of free HP without proline resulted in inhibited shoot formation despite elevated endogenous proline concentration, which was reversed by exogenous proline addition (Milazzo et al., 1998). In this present study free HP also inhibited shoot organogenesis, but the addition of FPH with HP reversed the inhibition, resulting in highly stimulated multiple shoot

formation correlating with high endogenous proline content. In the presence of FPH and HP, proline synthesis continued to occur and may continually stimulate the proline-linked PPP and the subsequent critical pathways related to cytokinin biosynthesis (purine synthesis). This stimulation therefore may be linked to enhanced multiple shoot formation (Figure 3).

The addition of TP and FPH resulted in the inhibition of multiple shoot formation, despite significantly higher endogenous proline levels compared to control (Figure 2). TP is known to be a substrate for PDH in *Escherichia coli*, where it is oxidized to *N*-formylcysteine (Deutch, 1992). Proline synthesis is increased with the addition of TP because TP is a simple competitive inhibitor of PDH, but it can be oxidized under certain conditions (Deutch, 1992). TP oxidation may be inhibited in the presence of proline when proline itself is oxidized. As previously suggested, TP in the presence of FPH may reflect the dual properties of competitive inhibition versus the oxidation of TP and therefore, by this, inhibition of BA-induced multiple shoot formation may occur (Milazzo et al., 1998). The proline-linked redox cycle may be offset by this, subsequently altering the PPP that is required for purine biosynthesis toward other pathways such as shikimate for auxin synthesis. Formation of callus tissue in the presence of TP supports this speculation. In conclusion, the stimulation of multiple shoot formation in melon is clearly associated with enhanced proline synthesis, which is likely the result of the conversion of precursors found in FPH to proline, which is further enhanced by the presence of proline analogues such as A2C and HP (Figure 2).

Proline synthesis requires $NADPH_2$ generated from the PPP. The subsequent recycling of NADP may stimulate the PPP, regenerating $NADPH_2$ and phosphorylated sugars such as ribose-5-phosphate for purine synthesis as observed in animal models (Phang, 1985; Figure 1). Our study strengthens the hypothesis that cytokinin (BA)-induced shoot organogenesis may be stimulated by proline synthesis, and this may be further enhanced through proline oxidation, with glutamate again being recycled for proline synthesis. FPH is therefore a natural source of proline and its precursors and may be able to stimulate purine synthesis for enhanced shoot formation by stimulating the proline-linked PPP. The $NADPH_2$-requiring steps during proline synthesis may be coupled to the PPP as suggested for soybean nodules and in animal models (Kohl et al., 1988; Phang, 1985). Multiple shoot formation (organogenesis) could also be stimulated by the enhanced natural endogenous cytokinins generated from purine metabolism as a direct result of the redox cycle generated by the proline-linked PPP.

As suggested previously, regulation of ribose-5-phosphate levels may be critical in determining how much is diverted to PRPP or erythrose-4-phosphate for purine and aromatic biosynthesis, respectively (Milazzo et al., 1998). The balance of natural cytokinins and auxin required for multiple shoot formation may be regulated by the modulation of purine and aromatic biosynthesis. Additionally, the stimulation of the proline-linked redox cycle may provide an alternative oxidative phosphorylation step using proline as a reductant instead of NADH (Phang, 1985; Rayapati and Stewart, 1991) during in vitro differentiation. Proline may be oxidized to initiate a redox cycle, and its further synthesis requires $NADPH_2$ generated from the PPP to complete its regeneration. Thus, deregulation of the PPP occurs and facilitates purine production from ribose-5-phosphate, thereby stimulating the production of natural cytokinins and enhancing BA-induced shoot organogenesis.

FPH provides an alternative to the addition of proline and proline precursors to stimulate BA-induced shoot organogenesis. FPH, a major byproduct of the fishery industry, the disposal of which causes environmental problems, can be utilized successfully for stimulating BA-induced shoot organogenesis for value-added products in the plant propagation industry.

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