Stimulation of Rosmarinic Acid in Shoot Cultures of Oregano (*Origanum vulgare*) Clonal Line in Response to Proline, Proline Analogue, and Proline Precursors

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Tissue culture-generated shoot-based clonal lines are being used to investigate the role of proline-linked pentose phosphate pathway in stimulating rosmarinic acid (RA). In this study is reported the stimulation of RA biosynthesis in oregano clonal line O-1 in response to proline, proline precursors (ornithine and arginine), and proline analogue (azetidine-2-carboxylate, A2C). Following exogenous treatment with proline and proline precursors in the presence or absence of proline analogue A2C, significantly enhanced RA content and concurrently higher levels of endogenous proline were observed compared to control. Analogue (A2C) treatment alone stimulated highest levels of RA without any increase in endogenous proline. Endogenous proline levels on day 30, however, were significantly higher in all proline and ornithine treatments with or without A2C but not in treatments with A2C or arginine alone. The stimulation of RA synthesis in response to proline or proline precursors with or without A2C suggested that deregulation and enhancement of proline synthesis or proline oxidation may be important for RA biosynthesis in oregano. This stimulation of RA biosynthesis provided strong clues that proline synthesis may be linked to stimulation of the pentose phosphate pathway, driving key precursor metabolites toward shikimate and phenylpropanoid pathways. RA-stimulating compounds also enhanced total phenolics and hardened stem tissues, indicating possible lignification due to polymerization of phenolic metabolites.

Keywords: *Arginine; azetidine-2-carboxylate; Origanum vulgare; ornithine; phenolics; proline; rosmarinic acid; shoot culture*

INTRODUCTION

Rosmarinic acid (α-*o*-caffeoyl-3,4-dihydroxyphenyllactic acid; RA), a plant secondary metabolite belonging to the class of hydroxycinnamic acid esters, is a prominent constituent of members of the Lamiaceae and Boraginaceae families (De-Eknamkul and Ellis, 1987). It has applications as a food preservative and in medicine due to its functional properties as an antioxidant and antimicrobial (Deighton et al., 1993; Madsen and Bertelsen, 1995; Frankel et al., 1996; Shetty et al., 1997). It was reported that in bulk corn oil rosemary extract containing carnosic acid and rosmarinic acid showed significantly more antioxidant activity than carnosol (Frankel et al., 1996). In addition, recent studies show that RA can also inhibit inflammatory reaction in vivo (Englberger et al., 1988; Peake et al., 1991). RA has also been reported to be accumulated in cultured cells of a number of species including *Coleus blumei* (Razzaque and Ellis, 1977; Zenk et al., 1977), *Anchusa officinalis* (De-Eknamkul and Ellis, 1984), *Lithospermum erythrorhizon* (Fukui et al., 1984), *Orthosiphon aristatus* (Sumaryono et al., 1991), and *Salvia officinalis* (Hippolyte et al., 1992). Even though RA can be produced in cell cultures, high RA content in leaf extracts of single seed origin Elite lines of the above species is beneficial for food applications and for enhancing the value of herb extracts for medicinal use. Therefore, we are studying RA synthesis in in vitro shoot cultures, which are being targeted for eventual production in greenhouse systems.

Early studies on the biosynthesis of RA (Ellis and Towers, 1970; Razzaque and Ellis, 1977) showed that phenylalanine and tyrosine are the respective precursors of the caffeoyl and 3,4-dihydroxyphenyllactic acid units of RA. Phenylalanine ammonia-lyase (PAL) catalyzes the initial step of the phenylpropanoid pathway leading to 4-coumaroyl CoA, whereas the entry point enzyme of the tyrosine-derived pathway was shown to be tyrosine aminotransferase (TAT) (De-Eknamkul and Ellis, 1987). Recent studies (Mizukami et al., 1992; Sumaryono et al., 1991) showed that the increase of RA content by yeast extract (YE) treatment coincided with the increase of PAL activity, indicating the important role of PAL in the regulation of RA biosynthesis. Other steps in RA biosynthesis from phenylalanine and tyrosine have also been characterized (Peterson et al., 1993). However, there is little understanding of the role of primary metabolism at the level of the pentose phosphate pathway for overall regulation of RA biosynthesis. These insights will help develop approaches for regulating RA biosynthesis for food and pharmaceutical applications.

The model for investigating the role of primary metabolism is that the proline-linked pentose phosphate pathway is critical for driving precursors (erythrose-4-phosphate) toward shikimate and phenylpropanoid

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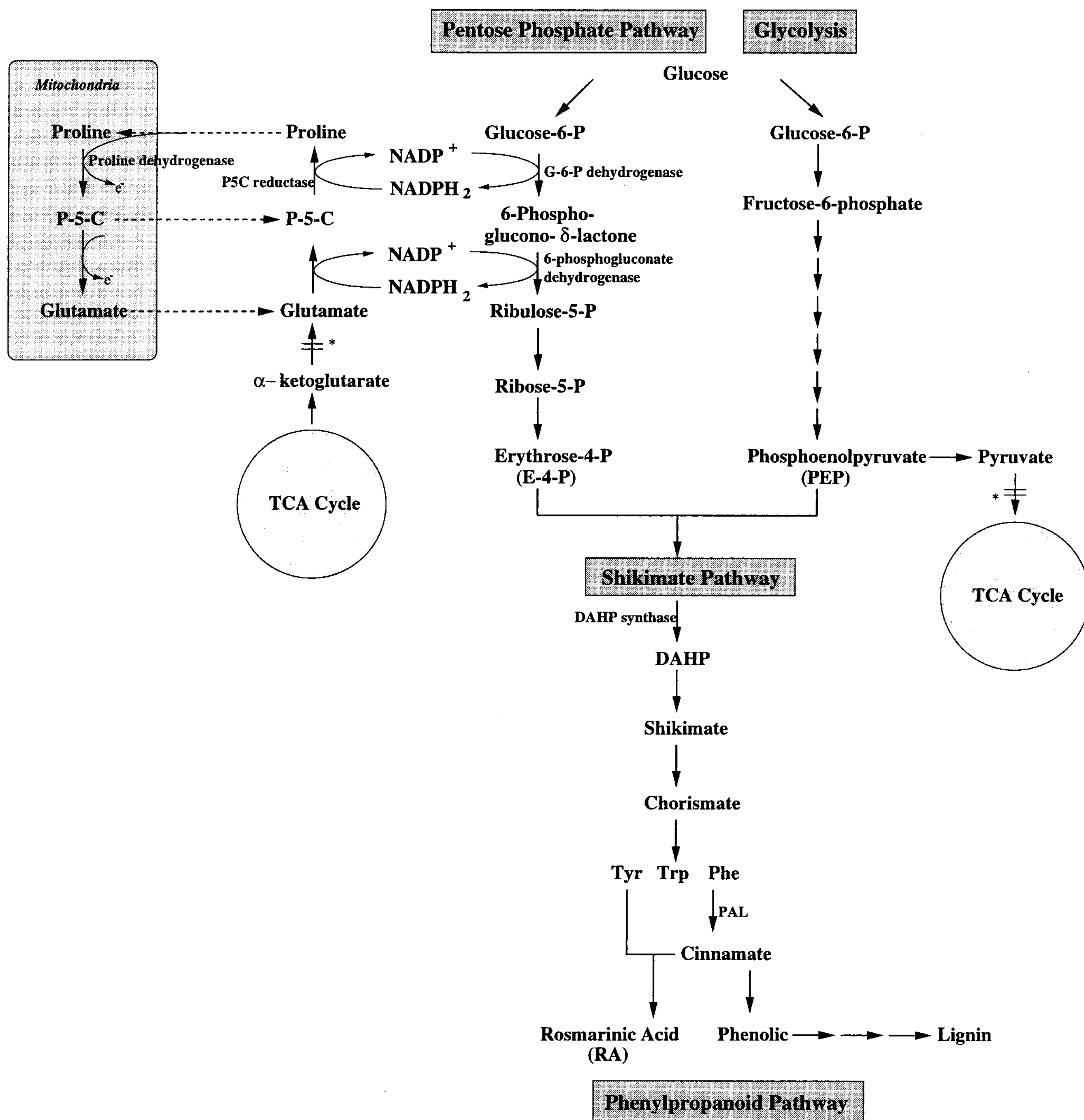

Figure 1. Model for role of proline-linked pentose phosphate pathway in stimulating total phenolics and RA. (*Reduced flux for α-ketoglutarate during phenolic and RA synthesis).

pathways (Figure 1). The rationale for this model is based on the role of the proline-linked pentose phosphate pathway in driving ribose-5-phosphate toward purine synthesis in cancer cells (Phang, 1985) and possibly in plant tissues (Kohl et al., 1988) as well as making available $NADPH_2$ for all anabolic reactions. The interconversion of ribose-5-phosphate to erythrose-4-phosphate is critical for shikimate and phenylpropanoid pathways, and it has to be derived from the pentose phosphate pathway (Schnarrenberger et al., 1995). The stimulation of a proline-linked redox cycle may drive the pentose phosphate pathway to make available $NADPH_2$ and ribose-5-phosphate; in addition, proline may serve as a reductant, replacing $NADH_2$ as the hydrogen donor for oxidative phosphorylation in the mitochondria (Phang et al., 1982; Phang, 1985; Rayapati and Stewart, 1991).

The long-term objective of our laboratory is to explore the role of the proline-linked pentose phosphate pathway in stimulating RA synthesis in shoot cultures of clonal lines in the family Lamiaceae. If the role of the proline-linked pentose phosphate pathway in the stimulation of RA synthesis is to be investigated, then the enhancement of RA synthesis by providing proline and proline precursors with proline analogues should be confirmed. The addition of a proline analogue, azetidine-2-carboxylate (A2C), should deregulate proline synthesis, which, if coupled to the pentose phosphate pathway at the level of glucose-6-phosphate dehydrogenase, should generate a redox cycle. This proline-

linked redox cycle, by stimulating synthesis of $NADPH_2$ and ribose-5-phosphate, should drive metabolite precursors toward shikimate and phenylpropanoid pathways (Figure 1). In this paper, we have investigated the effects of exogenous proline, proline analogue (A2C), and proline precursors (arginine and ornithine) on RA synthesis in genetically uniform shoot culture of an oregano (*Origanum vulgare*) clonal line. We also measured the phenolic content and tissue rigidity indicative of lignification in response to the above treatments.

MATERIALS AND METHODS

Shoot Culture and Treatment. Oregano shoot cultures of clonal line O-1 originating from a single heterozygous seed were previously isolated using auxilliary bud proliferation (Shetty et al., 1995). After 60-day shoot multiplication on Murshige and Skoog medium (Murashige and Skoog, 1962) with 1 mg/L benzyladenine, individual shoot apex explants of the O-1 clonal line were transferred to half-strength MSHF medium with different treatments, which include proline [P] (5 mM), A2C (50 μM), $P_{5.0}$ + $A2C_{50\mu M}$, arginine [Arg] (5 mM), $Arg_{5.0}$ + $A2C_{50\mu M}$, ornithine [Orn] (5 mM), and $Orn_{5.0}$ + $A2C_{50\mu M}$. The half-strength MSHF medium without any supplementation was used as control. On days 15 and 30, RA content and free proline content were measured.

RA Content. RA was estimated according to a modified spectrophotometric method originally described by López-Arnaldos et al. (1995). Approximately 100 mg (fresh mass) of explant was placed in 5 mL of 50% (v/v) methanol and allowed to incubate for 2 h in a 55 °C walk-in growth incubator. After the sample cooled to room temperature, exactly 1 mL of extract was transferred to a 16 × 100 mm test tube and diluted by the addition of 3 mL of 50% (v/v) methanol. The absorbance was then measured at 333 nm by spectrophotometer (Spectronic Genesys 5, Milton Roy Co., Rochester, NY), and the RA content was calculated and expressed as milligrams of RA per gram of fresh weight of plant tissue (ϵ = 19 000 L/mol·cm).

Proline Content. Free proline content was measured on the basis of a previously modified assay used for plants (Bates et al., 1973). Approximately 100 mg of oregano tissue was weighed and placed in 2 mL of 3% sulfosalicylic acid solution (Sigma Chemical Co., St. Louis, MO). After homogenization and centrifugation (13000*g*, 5−10 min) of the sample, 1 mL of supernatant was placed in a reaction test tube with a Teflon cap. Glacial acetic acid (1 mL; Fisher Scientific Co., Fair Lawn, NJ) and acid ninhydrin (1 mL; Sigma) were added to each tube, which is closed tightly later and heated in 100 °C water bath for 1 h. After the sample had cooled in an ice bath for ~15 min, 2 mL of toluene 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. Standard curves were prepared with each assay using standard proline (Sigma) in 3% sulfosalicylic acid solution. Proline content was expressed as micromoles per gram of FW of plant materials.

Total Phenolic Content. Total phenolic content was determined from a modified assay described by Chandler and Dodds (1983), which is similar to the method originally developed by Singleton and Rossi (1965) (Shetty et al., 1995). Approximately 50 mg (fresh weight) tissue samples were taken and placed in 2.5 mL of 95% ethanol. After samples had been stored at 0 °C for ~48−72 h, a Tissuemizer was used to homogenize oregano samples in ethanol. After centrifugation at 13000*g* for 10 min, exactly 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) was added to each sample. After 5 min, 1 mL of 5% Na_2CO_3 (Fisher) was added and mixed with a vortex mixer (Barnstead/Thermolyne, Dubuque, IA), 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. Standard curves were prepared with each assay using gallic acid in 95% ethanol. Total phenolic content was expressed as milligrams per gram of FW of plant material.

Table 1. Proline Content (Micromoles per Gram of FW ± SD)[a] of Oregano Clonal Line O-1 with Different Treatments

treatment	day 15	day 30
control	1.02 (0.29)	0.82 (0.25)
$P_{5.0mM}$	56.00 (22.61)	29.03 (19.90)
$A2C_{50\mu M}$	1.63 (0.52)	1.62 (0.84)
P + A2C	31.17 (8.16)	39.68 (19.14)
$Arg_{5.0mM}$	7.04 (1.52)	2.84 (0.83)
Arg + A2C	6.08 (1.11)	2.08 (0.94)
$Orn_{5.0mM}$	16.34 (4.52)	9.57 (3.02)
Orn + A2C	28.79 (5.75)	8.65 (5.29)

[a] Results are an average from two separate experiments with four replicates in each experiment.

Table 2. Rosmarinic Acid Content (Milligrams per Gram of FW ± SD)[a] of Oregano Clonal Line O-1 with Different Treatments

treatment	day 15	day 30
control	1.73 (0.67)	0.84 (0.61)
$P_{5.0mM}$	4.80 (0.77)	2.21 (0.56)
$A2C_{50\mu M}$	5.89 (1.50)	3.07 (0.53)
P + A2C	3.25 (1.49)	3.46 (1.00)
$Arg_{5.0mM}$	3.17 (0.84)	2.71 (0.78)
Arg + A2C	4.09 (1.25)	3.02 (0.61)
$Orn_{5.0mM}$	4.03 (1.32)	4.20 (0.84)
Orn + A2C	4.40 (2.15)	3.40 (0.65)

[a] Results are an average from two separate experiments with four replicates in each experiment.

Statistical Analysis. Analysis of data was done with Microsoft Excel for windows 95, version 7.0, 1985−1995 for Student *t*-test analysis of data for significance of difference between control and treatments.

Morphological Observations. Since proline, proline analogue, and proline precursor treatments were hypothesized to stimulate phenolics, the effect on visual morphological changes due to possible lignification was observed. Photographs were taken by Nikon camera with different exposures.

RESULTS AND DISCUSSION

Proline content and RA level of each treatment were measured on days 15 and 30. Total phenolic content was measured on day 30.

The basal level of proline content (Table 1) ranged from 1.02 to 0.82 μmol/g of FW on days 15 and 30. Similar to RA level (Table 2), it decreased with time (day 15 to day 30) except for A2C treatment, which had no significant decrease, and P + A2C, which showed an interesting increase on day 30.

Compared to control group, all treatments showed an increase in proline content. A2C treatment had the lowest proline content, which could mean no proline accumulation occurred. Precursors of proline, arginine, and ornithine had different but significant degrees of endogenous proline content. On the other hand, the increase of endogenous proline in response to exogenous P and P + A2C treatments is likely due to proline uptake from the medium.

The basal level of RA (control) was ~1.73 mg/g of FW on day 15 and decreased to 0.84 mg/g of FW on day 30. In general, there was a decrease in RA content on day 30 compared to day 15 except in response to P + A2C and ornithine treatments, for which the RA levels

Table 3. Total Phenolic Content (Milligrams per Gram of FW ± SD)[a] of Oregano Clonal Line O-1 with Different Treatments

treatment	day 30
control	1.85 (0.36)
$P_{5.0mM}$	2.74 (0.31)
$A2C_{50\mu M}$	2.93 (0.52)
P + A2C	3.02 (0.53)
$Arg_{5.0mM}$	2.92 (0.35)
Arg + A2C	2.38 (0.44)
$Orn_{5.0mM}$	3.07 (0.36)
Orn + A2C	3.33 (0.51)

[a] Results are an average from two separate experiments with four replicates in each experiment.

stayed relatively stable, from 3.25 to 3.46 mg/g of FW and from 4.03 to 4.20 mg/g of FW, respectively.

All treatments, compared to the control group, showed statistically significant increase in RA content. Among them, the A2C treatment had the highest increase (5.89 mg/g of FW) on day 15; in contrast, in response to arginine the lowest increase (3.17 mg/g of FW) was observed. On the other hand, on day 30 the ornithine treatment had the highest RA content (4.20 mg/g of FW).

In addition, all treatments showed significant increase in total phenolic synthesis on day 30 (Table 3). The basal level of phenolic content was ~1.85 mg/g of FW, and compared to control the other treatments had at least 29% (Arg + A2C) increase in total phenolic level. Corresponding with phenolic content increase, there was an increase in rigidity of shoot tissues in response to all treatments, which may indicate lignification (Figure

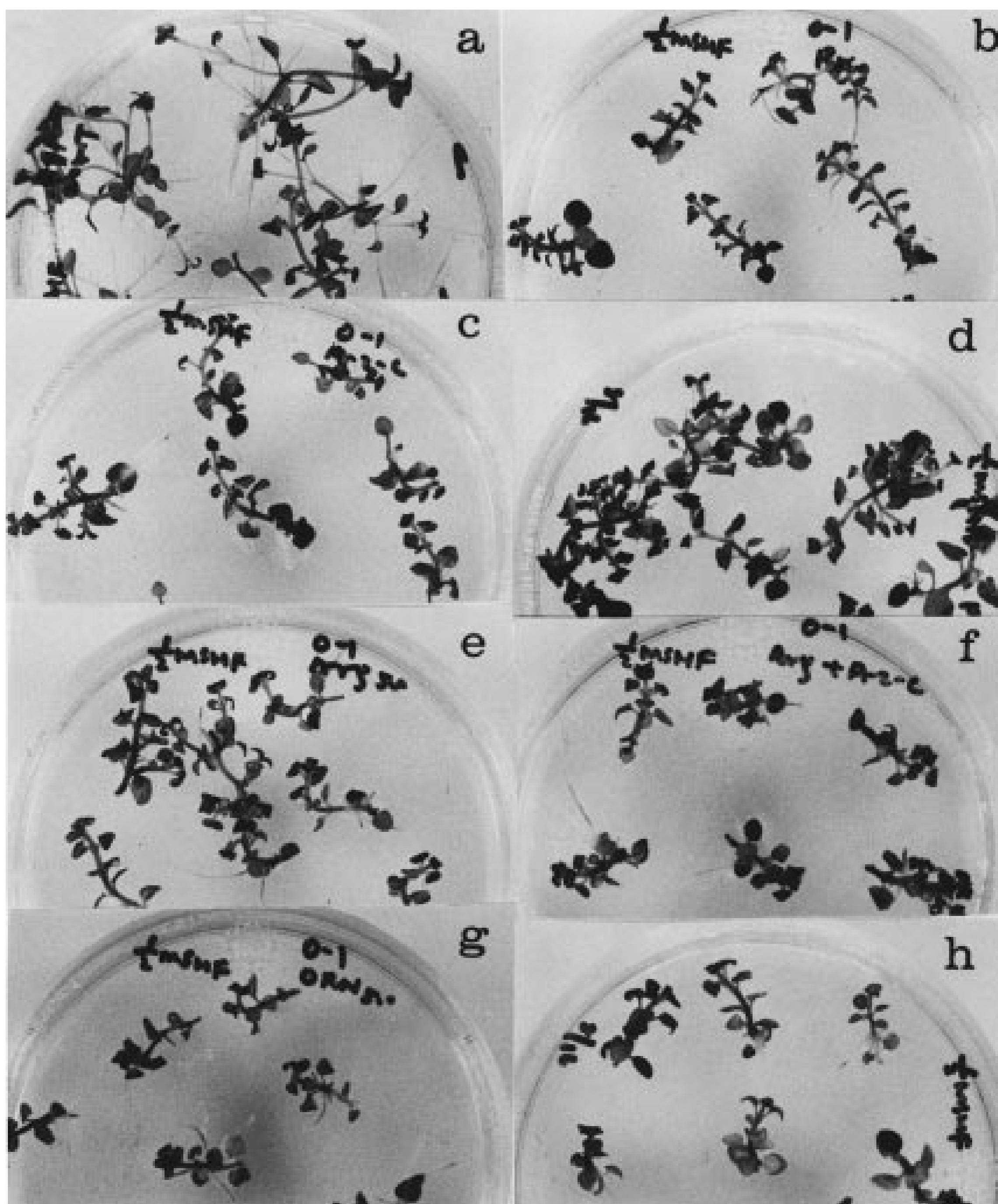

Figure 2. Response of oregano shoot clone O-1 to treatments: (a) control; (b) P5.0; (c) A2C; (d) P5.0 + A2C; (e) Arg5.0; (f) Arg5.0 + A2C; (g) Orn6.0 (h) Orn5.0 + A2C (note the tissue rigidification in treated shoots when compared to control).

2). We are currently undertaking studies to confirm lignification by specific chemical assays.

The increase in RA content in response to proline suggested that it is likely that proline oxidation may have occurred. If this were the case, proline oxidation could drive the pentose phosphate pathway as in erythrocytes (Phang et al., 1982) by recycling glutamic acid into the cytosol to generate a redox cycle. It was evident that by day 30 proline accumulation was reduced by half and likewise RA content. It is possible that RA content once formed maximally at early stages (day 15) was possibly oxidized by an RA peroxidase (López-Arnaldos et al., 1994) by day 30. The phenolic content and tissue rigidification in response to proline also increase, indicating a possible conversion of proline-stimulated metabolites toward lignification (Figure 2b).

Proline analogue A2C is a weak competitive inhibitor of proline for sites on proline-oxidizing enzyme proline dehydrogenase (PDH) (Elthon and Stewart, 1984). In the presence of A2C, RA was significantly stimulated without a corresponding increase in proline content. This strongly suggested that in the presence of A2C, proline may be continuously produced and immediately oxidized to out-compete A2C. This then favors the stimulation of the pentose phosphate pathway, which drives metabolites toward RA, phenolics, and lignification. The reduced RA on day 30 could again be due to the activity of RA peroxidase, which we will be investigating further in the future.

In response to proline + A2C, initially exogenous proline may be oxidized to out-compete A2C for PDH sites and therefore could stimulate RA synthesis by stimulating the pentose phosphate pathway. As shoot clones grow, by day 30 both new synthesis and oxidation may occur simultaneously. Proline analogue A2C may deregulate proline synthesis as proline content was reduced. This also could result in new RA synthesis and concurrent RA oxidation (Tables 1 and 2). The phenolic content and tissue rigidification in response to proline + A2C is significantly high (Table 3 and Figure 2d). This treatment further confirms that perturbing proline metabolism significantly stimulates the phenylpropanoid pathway.

Ornithine and arginine are not direct precursors on the pathway for proline biosynthesis (Csonka and Baich, 1983). These indirect proline precursors stimulated RA with a concurrent increase in proline content. This further provides support for a link of proline synthesis/oxidation to the pentose phosphate pathway which then stimulates the phenylpropanoid pathway and therefore enhances RA content, phenolic level, and lignification (Tables 1−3; Figure 2e,g). These effects are more pronounced when A2C was combined with the proline precursors arginine and ornithine.

In the presence of A2C, the proline precursors ornithine and arginine were forced to be converted to proline, which then helps to out-compete A2C at PDH sites. This enhances both proline synthesis and possibly proline oxidation (Table 1), with concurrent enhancement of RA (Table 2), phenolics (Table 3), and potential lignification (Figure 2f,h). This further strengthens our model that the proline-linked pentose phosphate pathway is important for redirecting metabolites toward shikimate and phenylpropanoid pathways (Figure 1).

CONCLUSION

In conclusion, it was clear that proline, proline analogue, and proline precursors clearly can stimulate RA synthesis in shoot cultures of oregano clonal line O-1. This strongly suggests that perturbing the proline metabolism can help to redirect metabolites from the pentose phosphate pathway toward total phenolic and RA synthesis (Figure 1). This indicates that the proline-linked pentose phosphate pathway may be operational and may be essential for making precursor intermediates for shikimate and phenylpropanoid pathways. On the basis of the studies in this paper, it is feasible to now study this potentially novel mode of regulation of phenolic secondary metabolites at the molecular level.

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