Tissue Culture Based Screening for Selection of High Biomass and Phenolic Producing Clonal Lines of Lavender Using *Pseudomonas* and Azetidine-2-carboxylate

Hussein Al-Amier,[†,‡] B. M. M. Mansour,[‡] N. Toaima,[‡] R. A. Korus,[§] and K. Shetty*,[†]

Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, Department of Horticulture, Faculty of Agriculture, Al-Azhar University, NasrCity, Cairo, Egypt, and Department of Chemical Engineering, University of Idaho, Moscow, Idaho 83843

Lavender is a good source of essential oils and phenolic metabolites for food, medicine, and cosmetic applications. Due to cross-pollination, lavender has substantial plant to plant variation and therefore a high degree of genetic inconsistency in the level of phytochemicals produced for diverse applications. Tissue culture methods, using benzyladenine-induced shoot organogenesis, were used to isolate clonal lines originating from individual heterozygous seeds among a heterogeneous seed population to exploit the genetic heterogeneity. Subsequently, in a two-step method, clonal shoots of each clonal line were evaluated for the ability to tolerate *Pseudomonas* inoculation and various levels (0−200 μM) of proline analogue, azetidine-2-carboxylate. On the basis of tolerance to *Pseudomonas* and proline analogue treatments, multiple shoot forming ability, biomass, rosmarinic acid, total phenolics, and total chlorophyll, 20 separate clonal lines were screened and isolated for further vegetative propagation and evaluation. From the clonal lines isolated, lines LH-14, LH-15, LH-17, and LH-11 showed the best potential for overexpression of phenolic metabolites in response to *Pseudomonas* and proline analogue.

Keywords: *Clonal lines; lavender; shoot culture; Pseudomonas tolerance; azetidine-2-carboxylate; phenolics; rosmarinic acid; high biomass*

INTRODUCTION

Lavender (*Lavandula angustifolia* Mill.), a member of the Lamiaceae (Labiatae) family, is widely used as a source of essential oils for perfumes, cosmetics, and botanical medicines (De Ginins Lassras, 1967; Piccaglia et al., 1993; Guilben and Cabo, 1996). It is also known that honey produced from areas near lavender fields have high concentrations of phenolic acids with antioxidant properties (Andrade et al., 1997). Lavender also contains antioxidant and anti-inflammatory compounds such as rosmarinic acid (López Arnaldos et al., 1994, 1995) with potential uses both as preventive medicines (Peake et al., 1991) and as food preservatives (Frankel et al., 1996). The challenge of harnessing the phytochemical potential of *L. angustifolia* L. is largely due to the substantial plant to plant heterogeneity resulting from the natural cross-pollination typical of species in the family Lamiaceae, including lavender. This results in highly variable phytochemical profiles for food, medicine, and other industrial uses. Using the strategy developed previously for other species in Lamiaceae such as thyme (Shetty et al., 1996), oregano (Eguchi et al., 1996), and rosemary (Yang et al., 1997), we have developed tissue culture based screening techniques to isolate lavender clonal lines with consistent phenolic phytochemical profiles. Our first strategy was to screen high phenolic stimulated clonal lines based on tolerance to *Pseudomonas* sp. (Eguchi et al., 1996; Shetty et al., 1996; Yang et al., 1997). The rationale for this screening approach is that phenolic metabolites are known to be stimulated in response to microbial elicitors (Dixon and Paiva, 1995), and any clonal line that is tolerant to *Pseudomonas* must be a phenolic overexpressing phenotype. Furthermore, in previous studies it was observed that the *Pseudomonas* challenge stimulated proline and the phenolic metabolite, rosmarinic acid (Perry and Shetty, 1998). Therefore, we hypothesized that the proline synthesis may be linked to the pentose phosphate pathway to concurrently provide sugar phosphates such as erythrose-4-phosphate for the shikimate pathway and subsequently the phenylpropanoid pathway (Figure 1). Proline and proline precursor ornithine in the presence of the proline analogue azetidine-2-carboxylate (A2C) were subsequently used to stimulate total phenolic and rosmarinic acid synthesis in oregano (Yang and Shetty, 1998). On the basis of these studies we hypothesized that tolerance to A2C could be effectively used as a secondary screen to isolate high phenolic producing clonal lines concurrently or following the *Pseudomonas* screen. The rationale for this secondary screen is that clonal lines tolerant to the proline analogue (A2C) would have to produce high proline levels, which would have to be immediately oxidized by mitochondrial-linked proline dehydrogenase to outcompete A2C. Clonal lines with this ability could therefore have a preferential proline-linked alternative oxidative phosphorylation for energy (ATP) synthesis as well as a proline synthesis-linked redox cycle that could stimulate the pentose phosphate pathway to efficiently produce $NADPH_2$ and sugar phosphates such as erythrose-

* Corresponding author [telephone (413) 545-1022; fax (413) 545-1262; e-mail kalidas@foodsci.umass.edu].

† University of Masschusetts.

‡ Al-Azhar University.

§ University of Idaho.

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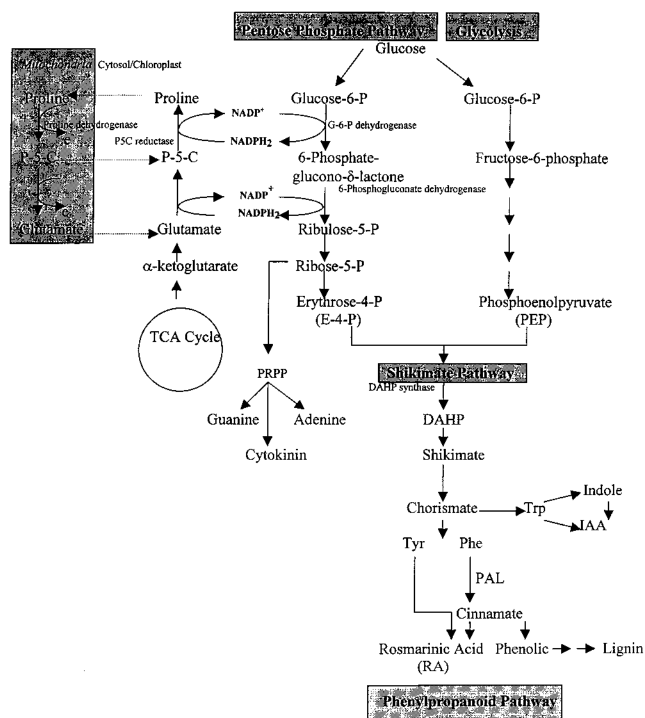

Figure 1. Model for role of proline-linked pentose phosphate pathway in stimulating total phenolics and RA.

4-phosphate for anabolic pathways (Phang, 1985; Shetty, 1997). In this paper we report a two-step, *Pseudomonas* and proline analogue (A2C) based screening strategy to isolate phenolic stimulated clonal lines of lavender with each line originating from different heterozygous seeds among a heterogeneous bulk seed population.

MATERIALS AND METHODS

Plant Material. Lavender shoot explants, each originating from a different heterozygous seed, contained an apical bud and one adjacent leaf pair. These explants were induced to produce multiple shoots via adventitious shoot formation on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) to enlarge the clonal plants (Shetty et al., 1995). Each clonal line originated from a single heterozygous seed among a heterogeneous bulk seed population. Initially 200 heterozygous seeds were aseptically germinated. From this pool of 200 seeds only ~20 heterozygous seedlings had the ability to form multiple shoots under the influence of benzyladenine (benzylaminopurine).

Media Preparation. Lavender explants of each clonal line were induced to produce multiple shoots on MS medium (Murashige and Skoog, 1962) containing 6-benzylaminopurine (BAP) and maintained on the same medium. The MSBAP medium contained 4.33 g/L MS basal salts mixture (Sigma Chemical Co., St. Louis, MO), 1 mL/L Nitsch and Nitsch vitamin solution diluted from a 1000× stock (Sigma Chemical Co.), 1 mL/L BAP (Sigma Chemical Co.), 30 g/L sucrose (Domino Sugar Corp., New York), and 3 g/L phytagel (Sigma Chemical Co.) and was made up to 1 L with distilled deionized water. The final pH of the medium was adjusted to 5.8. The solution was then sterilized by autoclaving at 120 °C and 15 lb of pressure for 20 min. For experiments, individual shoots of each clonal line were transferred from MSBAP medium to a half-strength MS hormone-free (half-MSHF) medium. This medium contained 2.17 g/L MS basal salt mixture, 0.5 mL/L Nitsch and Nitsch vitamin solution from 1000× stock, 15 g/L sucrose, and 3 g/L Phytagel and was made up to 1 L with distilled deionized water. The pH of solution was adjusted to 5.8 and the solution was sterilized. The half-strength MSHF medium contained 0−200 μM proline analogue A2C.

***Pseudomonas* Species and Maintenance.** *Pseudomonas mucidolens* (ATCC 4685) was obtained from the American Type Culture Collection and used for hyperhydricity reduction studies in oregano (Ueno and Shetty, 1997, 1998). The bacteria were maintained on yeast mannitol agar (YMA) medium (Difco Laboratories, Detroit, MI). *P. mucidolens* inoculated on YMA medium was incubated at room temperature, 21−23 °C, for 72 h and then stored at 2 °C until needed. Subculturing was done every 3 months for new inoculation stock, for 2 °C storage, and for routine shoot inoculation.

Inoculation of Lavender Shoot Explants. Individual shoots of each lavender clonal line were inoculated with the *P. mucidolens* (ATCC 4685) by dipping cut shoot explants onto bacterial colonies on YMA plates and transferred to half-strength basal salt, hormone-free MS medium (Shetty et al., 1995).

Each clonal line for A2C treatment or *P. mucidolens* treatment had similar numbers of replicates of uninoculated treatment as controls. Every treatment had five replicates (five Petri dishes), and each replicate had seven explants. The Petri plates containing shoot apex explants were incubated at 26 °C with a 16 h light cycle and a light intensity of 40 μmol m^{-2} s^{-1}; the key parameters were measured after 30 days.

Biomass Parameters. Biomass parameters were taken after 30 days from treatment to measure the ability of clonal lines to produce biomass (fresh weight) and tolerance to *P. mucidolens* and A2C. Survival percent, number of shoots per explant, and fresh weight of explants were measured using a Sartorius balance (model BA 110, Denever Instrument Co., Avada, CO).

Total Phenolics Assay. Total phenolics were determined essentially according to the method of Chandler and Dodds (1983) based on that of Singleton and Rossi (1965). Approximately 50 mg (fresh mass) of shoot tips was placed in 2.5 mL of 95% ethanol and held at 0 °C for 48 h. Each sample was then homogenized with a Tissue Tearor (Biospec. Product, Racine, WI) and centrifuged at 13000*g* for 8−10 min. One milliliter of supernatant was transferred to a 16 × 100 mm test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample were added 0.5 mL of 50% Folin−Ciocalteu reagent and, after 5 min, 1 mL of 5% Na_2C0_3, and the samples were mixed and allowed to stand for 60 min. The absorbance was read at 725 nm with 95% ethanol as blank using a Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY). A standard curve was established using various concentrations of gallic acid in 95% ethanol. Absorbance values were converted to milligrams of total phenolics per gram of fresh tissue. This method is a well-established assay to estimate total hydroxylated phenolics, both monophenolics and flavonoids.

Rosmarinic Acid (RA) UV Assay. The UV assay for RA was modified from López-Arnaldos et al. (1995). Approximately 50 mg (fresh weight) tissue samples were placed in 3 mL of 50% methanol (diluted with distilled deionized water to 50% v/v), and samples were incubated at 55 °C for 2 h. One milliliter of the methanol extract was taken and diluted with 5 mL of methanol. Fifty milligrams per 18 mL of the diluted extracts was vortexed, and absorbance was measured at 333 nm. Concentration of RA in diluted extract was calculated using $A = \epsilon bc$, where the extinction coefficient ϵ = 19000 L/mol·cm and the width of disposable quartz cuvettes b = 1 cm. This method has been found to be accurate for routine, large-scale analysis of RA and has been found comparable to the HPLC method (López-Arnaldos et al., 1995).

Chlorophyll Content. Total chlorophyll was estimated according to the spectrophotometric method described by Hipkins and Baker (1986). Approximately 50 mg (fresh mass) of explant was placed in 3 mL of 100% methanol in 5 mL vials. The vials were covered and incubated at 23 °C for 2 h in darkness. Each sample was mixed, the methanol fraction decanted, and the absorbance measured at 650 and 665 nm. Total chlorophyll was expressed as micrograms per milliliter of methanol as follows:

$$\text{total chlorophyll} = 25.8A_{650} + 4.0A_{665}$$

A_{650} and A_{665} are absorbances at 650 and 665 nm, respectively. Total chlorophyll was then converted to micrograms of chlorophyll per gram of tissue as follows:

$$\mu\text{g of chlorophyll/mL of methanol} \times 3 \text{ mL of methanol/(g of tissue)}$$

Statistical Analysis. All experiments were five replicates (Petri dishes) per treatment and seven explants per replicates (Petri dish). SAS PROC.GLM was used with the Duncan multiple-range *t* test to determine least significant differences (LSD).

RESULTS

There was a high degree of variability among various multiple shoot forming heterozygous clonal lines. This variability was classified on the basis of two broad categories of response parameters following *Pseudomonas* and A2C treatments: (a) Biomass response included percent survival, multiple shoots per explant, and fresh weight per explant. (b) Metabolite response included RA, total phenolics, and total chlorophyll content. In all, 20 benzyladenine-induced multiple shoot forming individual clonal lines were isolated from 200 heterogeneous seeds. These 20 heterogeneous clonal lines were categorized into 8 general classes of clonal lines based on tolerance to *Pseudomonas* and A2C (Table 1). Overall, 50% (10) of the 20 multiple shoot forming clonal lines were tolerant to *Pseudomonas* and A2C concentration in the range 100−200 μM (Table 2). In general, all *Pseudomonas* and A2C tolerant clonal lines have increased phenolic and/or rosmarinic acid content in response to both treatments when compared to untreated control of the same clonal line. In contrast, generally most *Pseudomonas* susceptible lines, except those tolerant to 200 μM A2C, do not have phenolic or RA increases in response to A2C. Even lines tolerant to 200 μM A2C have moderate stimulation of phenolics or RA in response to this higher level of A2C.

Another interesting observation was that >50% of the *Pseudomonas* and A2C tolerant clonal lines have higher fresh weight per explant (i.e., >0.1 mg) in untreated control. Overall, biomass (fresh weight) decreased following treatments, except for LH-9 (no change) and LH-14 (increased) in response to *Pseudomonas* and LH-1, LH-3, LH-10, and LH-12 in response to 50 μM A2C. Likewise, multiple shoot forming ability was reduced in response to treatments except LH-5 and LH-9 in response to *Pseudomonas* as well as LH-1 and LH-10 in response to 50 μM A2C. Multiple shoot forming ability was the highest among clonal lines LH-3, LH-5, LH-9, LH-11, and LH-13. Interesting was the fact that, except for LH-3, all other clonal lines with high multiple shoot forming ability were *Pseudomonas* tolerant. The *Pseudomonas* tolerance based on survival percent was between 50 and 70%.

Among metabolite parameters, RA and total phenolic stimulation were the best indicators of *Pseudomonas* and A2C tolerance. Basal RA level was highest in LH-17, LH-8, LH-15, and LH-11 corresponding to RA of 2.5, 2.7, 3.5, and 4.4 mg/g of FW, respectively. All of these clonal lines were *Pseudomonas* tolerant, with LH-8 (141%), LH-15 (125%), and LH-17 (133%) having very significant RA increases in response to *Pseudomonas* compared to untreated control and LH-11 having no increase. In untreated explants basal phenolic levels of

Table 1. Categories of *Pseudomonas* and A2C Tolerant Clonal Lines of Lavender

Pseudomonas tolerance	A2C tolerance level, μM A2C	clonal lines
tolerant lines	0	LH-16
	50	LH-6
	100	LH-8, LH-11, LH-12, LH-13, and LH-14
	200	LH-4, LH-5, LH-9, LH-15, and LH-17
susceptible lines	0	LH-18 and LH-20
	50	LH-2 and LH-19
	100	LH-3 and LH-10
	200	LH-1 and LH-7

>2 mg/g of FW were observed among clonal lines LH-10, LH-11, and LH-19. Among these lines only LH-11 was *Pseudomonas* tolerant with the ability to produce the highest (3.32 mg/g of FW) phenolics in response to the treatment. Clonal lines LH-2, LH-3, LH-5, LH-7, LH-9, LH-15, LH-17, LH-18, and LH-20 had basal phenolic levels in the range > 1 < 2 mg/g of FW. Among these lines, five lines (LH-2, LH-3, LH-7, LH-18, and LH-20) were *Pseudomonas* susceptible. There were eight clonal lines (LH-1, LH-4, LH-6, LH-8, LH-12, LH-13, LH-14, and LH-16) with basal phenolics <1 mg/g of FW. Among these lines, except for LH-1, all lines were tolerant to *Pseudomonas*. It was clear that phenolic stimulation was needed for *Pseudomonas* tolerance, and this was better facilitated with lower background (basal control) level.

A2C treatment response of clonal lines tolerant to *Pseudomonas* was determined in the range of 50−200 μM. Clonal line LH-6 was tolerant to 50 μM A2C and had low basal levels of RA (0.6 mg/g of FW) and phenolics (0.7 mg/g of FW). These levels were marginally stimulated in response to 50 μM A2C (Table 3). A total of 10 of the 20 clonal lines isolated were tolerant to A2C levels of 100 μM (5 clonal lines) and 200 μM (5 clonal lines). Clonal lines tolerant to 100 μM A2C had basal RA in the range of 1.3−4.4 mg/g of FW, which were stimulated in response to A2C in clonal line LH-8 (75%), LH-12(82%), and LH-14 (341%) but not in LH-11 (low stimulation) and LH-13. The basal phenolics in these A2C (100 μM) tolerant lines were in the range of 0.7−2.1 mg/g of FW and were stimulated in response to A2C in clonal line LH-8 (38%, moderate), LH-13 (157%), and LH-14 (133%) but not substantially stimulated in LH-11 (24%) and LH-12 (26%). Clonal lines tolerant to 200 μM A2C had basal RA in the range of 1.3−3.5 mg/g of FW and were stimulated in response to 200 μM A2C in clonal lines LH-4 (164%) and LH-17 (111%) but not in LH-5, LH-9, and LH-15. Line LH-5 was stimulated moderately (24%) in response to 100 μM A2C and line LH-15 at 50 μM A2C (66%). The basal phenolic levels ranged from 0.9 to 1.6 mg/g of FW and were stimulated in response to 200 μM A2C in LH-4 (22%), LH-5 (103%), and LH-15 (29%) but not in LH-9, and LH-17. Phenolic stimulation in LH-5 was pronounced but not in LH-4 and LH-15 (pronounced [106%] only in response to 100 μM A2C).

Pseudomonas susceptible clonal lines were tolerant to A2C in the range of 50−200 μM. The clonal lines LH-2 and LH-19 were tolerant to 50 μM A2C. In LH-2, RA was not stimulated in response to 50 μM A2C, and in LH-19 it was moderately stimulated (Table 3). Phenolic content was not stimulated in both clonal lines, and in fact in LH-19 it was substantially decreased. Clonal lines LH-3 and LH-10 were tolerant to 100 μM A2C.

Table 2. Metabolite Levels (RA, Total Phenolics, and Total Chlorophyll) Biomass Content in Response to *Pseudomonas* Treatment of Various Lavender Clonal Lines Originated from Different Heterozygous Seeds

clone	treatment	survival %	shoot no.	FW of expt[a]	RA,[b] mg/g	total phenolics,[b] mg/g	total chlorophyll,[b] μg/g
LH-1	control	100	5.5	0.07	2.1	0.9	241
	Pseudomonas	0					
LH-2	control	100	3.3	0.04	1.7	1.57	148
	Pseudomonas	0					
LH-3	control	100	8.5	0.06	1.5	1.58	115
	Pseudomonas	0					
LH-4	control	100	6.3	0.1	1.2	0.87	181
	Pseudomonas	61	3.3	0.04	1.8	1.01	168
LH-5	control	100	7.8	0.12	2.3	1.27	338
	Pseudomonas	64	7	0.09	1.9	1.04	321
LH-6	control	100	8.3	0.11	0.6	0.69	366
	Pseudomonas	61	4.3	0.05	2.1	1.85	314
LH-7	control	100	4.3	0.05	2.5	1.95	329
	Pseudomonas	0					
LH-8	control	100	4	0.07	2.7	0.91	384
	Pseudomonas	71	3.3	0.05	6.4	1.28	284
LH-9	control	100	7.8	0.09	2.4	1.62	517
	Pseudomonas	50	7	0.1	3.8	2.48	305
LH-10	control	100	4	0.04	1.8	3.86	338
	Pseudomonas	0					
LH-11	control	100	9.5	0.11	4.4	2.13	439
	Pseudomonas	68	3.8	0.04	3.6	3.32	406
LH-12	control	100	6.5	0.09	1.9	0.93	484
	Pseudomonas	61	3.8	0.05	1.6	1.11	245
LH-13	control	100	8.5	0.18	1.5	0.83	316
	Pseudomonas	64	3.5	0.04	3.1	0.85	516
LH-14	control	100	5.3	0.11	1.3	0.71	189
	Pseudomonas	64	3.3	0.26	5.1	1.57	336
LH-15	control	100	5	0.07	3.6	1.01	248
	Pseudomonas	68	3.3	0.04	7.8	3.04	266
LH-16	control	100	5	0.07	2.2	0.58	229
	Pseudomonas	71	4	0.05	0.9	1.7	245
LH-17	control	100	5.3	0.08	2.5	1.21	237
	Pseudomonas	50	3	0.04	5.8	2.96	199
LH-18	control	100	4.3	0.09	1.5	1.18	184
	Pseudomonas	0		0.09			
LH-19	control	100	5.3	0.09	1.8	2.8	369
	Pseudomonas	0					
LH-20	control	100	6	0.06	1.1	1.73	226
	Pseudomonas	0					
LSD at 5%	for clones	7	1	0.01	0.5	0.45	75
	for *Pseudomonas*	3	0.4	0.05	0.2	0.17	28

[a] Fresh weight (FW) measurement made on day 30. [b] All measurements based on fresh weight (FW).

The RA was not stimulated in response to A2C. The phenolic content was also not stimulated either in LH-3 or in LH-10, and in LH-10 it was substantially reduced. Clonal lines LH-1 and LH-7 were tolerant to 200 μM A2C. In LH-1, RA was not stimulated in response to A2C but phenolic levels were stimulated moderately. In LH-7, 100 μM A2C stimulated RA (68%), whereas 100–200 μM A2C stimulated total phenolic content (58%). In general, *Pseudomonas* susceptible lines did not have enhanced RA or phenolic content in response to A2C or were minimally stimulated as in the case of LH-7.

There was no consistent pattern in the extent of chlorophyll synthesis in response to *Pseudomona*s and A2C treatments. Basal chlorophyll levels of ~330 μg/g FW were observed in clonal lines LH-5, LH-6, LH-7, LH-8, LH-9, LH-10, LH-11, LH-12, LH-13, and LH-19. Among these, LH-7, LH-10, and LH-19 were susceptible to *Pseudomonas* and all other lines were *Pseudomonas* tolerant. Among these lines, chlorophyll stimulation in response to *Pseudomonas* was observed only in clonal line LH-13 (Table 2). All other lines had no change or had decreased chlorophyll levels. Clonal lines with chlorophyll levels in the range of 115–330 μg/g of FW range were equally categorized between *Pseudomonas* tolerant and susceptible groups. Among these tolerant lines, only clonal line LH-14 had a significant increase in chlorophyll content in response to *Pseudomonas*, whereas all other lines had no change or had marginal decreases (Table 2). Chlorophyll stimulation did not occur in response to A2C treatment, except for LH-3, LH-5, LH-13, and LH-14 at 50, 100, 100, and 50–100 μM, respectively. Chlorophyll stimulation was observed in LH-13 and LH-14 in response to both *Pseudomonas* and A2C treatment (Table 3).

DISCUSSION

An ideal lavender clonal line is one that would have (a) high multiple shoot forming ability with the highest biomass per explant, (b) no reduction or marginal reduction in biomass in response to *Pseudomonas* and A2C, and (c) increased stimulation of metabolites such as rosmarinic acid, total phenolics, and chlorophyll in response to *Pseudomonas* and A2C. High multiple shoot forming ability provides increased number of clonal propagules for rapid vegetative propagation on a large scale. *Pseudomonas* and A2C tolerant clonal lines that specifically have the potential to divert metabolites via the proline-linked pentose phosphate pathway (Phang, 1995; Shetty, 1997) would also have the potential to efficiently use proline as a reductant for oxidative phosphorylation (ATP synthesis). At the same time

Table 3. Metabolite Levels (RA, Total Phenolics, and Total Chlorophyll) Biomass Content in Response to A2C Treatments of Various Lavender Clonal Lines Originated from Different Heterozygous Seeds

clone	treatment	survival %	shoot no.	FW of expt[a]	RA,[b] mg/g	total phenolics,[b] mg/g	total chlorophyll,[b] μg/g
LH-1	control	100	5.5	0.07	2.1	0.9	241
	50 μM A2C	96	5.5	0.07	2.7	0.99	216
	100 μM A2C	86	3.8	0.06	1.8	1.21	211
	200 μM A2C	57	3.3	0.04	2	1.3	198
LH-2	control	100	3.3	0.04	1.7	1.57	148
	50 μM A2C	61	3.5	0.04	1.5	1.56	176
	100 μM A2C	0					
	200 μM A2C	0					
LH-3	control	100	8.5	0.06	1.5	1.58	115
	50 μM A2C	82	6	0.06	1.7	1.57	217
	100 μM A2C	46	3	0.04	1	1.65	178
	200 μM A2C	0					
LH-4	control	100	6.3	1	1.2	0.87	181
	50 μM A2C	71	4.5	0.06	2.9	1.08	228
	100 μM A2C	57	3.8	0.05	1.7	0.93	128
	200 μM A2C	54	3.5	0.06	3.3	1.06	338
LH-5	control	100	7.8	0.12	2.3	1.27	168
	50 μM A2C	96	4.5	0.09	1.8	1.31	308
	100 μM A2C	82	3.5	0.05	2.8	2.59	432
	200 μM A2C	50	2.8	0.03	1.9	2.24	134
LH-6	control	100	8.3	0.11	0.6	0.69	366
	50 μM A2C	64	4	0.05	1.3	0.9	283
	100 μM A2C	0					
	200 μM A2C	0					
LH-7	control	100	4.3	0.05	2.5	1.95	329
	50 μM A2C	79	3.5	0.04	2.3	2.44	330
	100 μM A2C	54	3.3	0.03	4.2	2.45	142
	200 μM A2C	43	2.8	0.03	2	3.09	127
LH-8	control	100	4	0.05	2.7	0.91	384
	50 μM A2C	79	3.3	0.05	5.8	1.19	367
	100 μM A2C	75	3.3	0.05	6.4	1.27	380
	200 μM A2C	0					
LH-9	control	100	7.8	0.09	2.4	1.62	517
	50 μM A2C	64	4.8	0.05	2.5	1.69	434
	100 μM A2C	57	3.8	0.06	2.9	1.89	309
	200 μM A2C	38	3.8	0.03	1.7	1.35	418
LH-10	control	100	4	0.04	1.8	3.86	338
	50 μM A2C	64	4.4	0.05	2.3	1.86	188
	100 μM A2C	50	3.8	0.04	2.3	1.93	322
	200 μM A2C	0					
LH-11	control	100	9.5	0.11	4.4	2.13	439
	50 μM A2C	61	4	0.03	5.7	3.45	307
	100 μM A2C	18	2.5	0.03	4.5	2.65	127
	200 μM A2C	0					
LH-12	control	100	6.5	0.09	1.9	0.93	484
	50 μM A2C	79	4.5	0.08	2	0.85	320
	100 μM A2C	57	3.8	0.07	3.5	1.17	400
	200 μM A2C	0					
LH-13	control	100	8.5	0.18	1.5	0.83	352
	50 μM A2C	86	6.8	0.14	1.3	0.85	270
	100 μM A2C	54	5.8	0.12	1.3	2.14	477
	200 μM A2C	0					
LH-14	control	100	5.3	0.11	1.3	0.71	189
	50 μM A2C	54	4.5	0.1	3.5	0.49	368
	100 μM A2C	50	3.8	0.08	5.7	1.58	257
	200 μM A2C	0					
LH_15	control	100	5	0.07	3.5	1.01	248
	50 μM A2C	93	4	0.06	5.5	1.68	183
	100 μM A2C	71	3.8	0.05	3.3	2.08	243
	200 μM A2C	68	3.5	0.06	3.1	1.31	168
LH-16	control	100	5	0.07	2.2	0.58	229
	50 μM A2C	0					
	100 μM A2C	0					
	200 μM A2C	0					
LH-17	control	100	5.3	0.08	2.5	1.215	237
	50 μM A2C	71	4.3	0.07	2.4	0.958	241
	100 μM A2C	57	3.5	0.06	3.1	0.844	227
	200 μM A2C	57	2.8	0.05	5.2	0.932	202
LH-18	control	100	4.3	0.09	1.5	1.185	184
	50 μM A2C	0					
	100 μM A2C	0					
	200 μM A2C	0					
LH-19	control	100	5.3	0.09	1.8	2.808	369
	50 μM A2C	68	4.5	0.08	2.8	0.847	227
	100 μM A2C	0					
	200 μM A2C	0					
LH-20	control	100	6	0.06	1.1	1.734	226
	50 μM A2C	0					
	100 μM A2C	0					
	200 μM A2C	0					
LSD at 5%	for clones	7	1	0.01	0.5	0.45	75
	for A2C	3	0.4	0.06	0.2	0.19	31

[a] Fresh weight (FW) measurement made on day 30. [b] All measurements based on fresh weight (FW).

proline oxidation could provide a redox cycle in the cytosol or chloroplast linked to mitochondrial respiration and generate $NADPH_2$ and sugar phosphates for anabolic pathways, including shikimate pathway, purine synthesis, and carbon fixation (in chloroplast).

From the lavender clonal lines generated through this study, lines LH-14 and LH-15 were potentially ideal for production of phenylpropanoid metabolites using tissue culture regenerated plant clonal systems. Line LH-14 produced an average of five multiple shoots per explant, which was reduced to three per explant in response to *Pseudomonas*. However, fresh weight per explant increased to 0.26 g in response to *Pseudomonas* from the background level of 0.11 g/explant. Furthermore, RA was stimulated from the background level of 1.3−5.1 mg/g of FW (293%) in response to *Pseudomonas*. Likewise, total phenolics (from 0.71 to 1.57 mg/g of FW) and total chlorophyll (from 189 to 336 μg/g of FW) were stimulated in response to *Pseudomonas*. A2C tolerance was moderate (100 μM) for the LH-14 line. In response to 100 μM A2C multiple shoot formation and biomass (FW) were marginally reduced from 5 to 3.8 and from 0.11 to 0.08 g/explant, respectively. RA and total phenolics were stimulated at 100 μM A2C, but total phenolics was inhibited at 50 μM A2C, during which treatment chlorophyll was highly stimulated (from 189 to 368 μg/g of FW).

Among other clonal lines, LH-15 and LH-17 also have potential for high overexpression of the phenylpropanoid pathway. The multiple shoot forming abilities of these lines were similar to that of LH-14, but reduction of basal biomass (FW)/explant in response to *Pseudomonas* was higher. LH-15 and LH-17 lines had higher basal RA and total phenolics than LH-14. These metabolites were substantially stimulated in response to *Pseudomonas* with RA in the range 6−8 mg/g of FW and phenolics in the range of 3 mg/g of FW. Chlorophyll content did not significantly change in response to *Pseudomonas*. Both LH-15 and LH-17 had higher tolerance to A2C than LH-14 with marginal reduction in biomass (Table 3). RA in LH-15 was stimulated in response to 50 μM A2C but was similar to untreated control at 100−200 μM levels. Total phenolics were stimulated at 50 μM A2C but were reduced at 200 μM. Chlorophyll levels initially were reduced at 50 μM but then increased at 200 μM. Line LH-17 had a concentration-dependent stimulation of RA in the 50− 200 μM range of A2C. Phenolics, however, were marginally reduced, and no significant changes in chlorophyll content were observed (Table 3).

Clonal lines LH-11 and LH-5 are very interesting lines. LH-11 was *Pseudomonas* and A2C (100 μM) tolerant and had the highest multiple shoot forming ability (9.5/explant). The biomass and multiple shoot forming ability were reduced by >50% in response to *Pseudomonas* with significant increase in total phenolics from 2.13 to 3.45 mg/g of FW. The RA and chlorophyll contents were reduced marginally. The A2C tolerance of LH-11 was clear at 50 μM, and in response to the treatment both biomass and multiple shoot forming ability significantly decreased; however, significant increases in RA and total phenolics were observed. Chlorophyll level was marginally reduced. Line LH-5, on the other hand, was highly tolerant to *Pseudomonas*, with no reduction in multiple shoot forming ability and marginal reduction in biomass. Among metabolites, the RA, total phenolics, and chlorophyll did not significantly change or were marginally reduced. An interesting observation was that this line was highly fragrant in response to *Pseudomonas* with a very distinct lavender aroma in the in vitro shoot cultures. A2C tolerance of LH-5 was high, with significant proportionate reduction in multiple shoots and biomass in response to A2C. RA, total phenolics, and chlorophyll were stimulated at 100 μM but not at 50 μM. At 200 μM A2C, only total phenolics was stimulated and RA and chlorophyll were significantly reduced.

The above clonal lines (LH-14, LH-15, LH-17, LH-11, and LH-5) provide only a sample of potential elite phytochemical profile-producing lavender lines from the original heterogeneous background for large-scale production and evaluation in greenhouse and field systems. The two-step strategy outlined in this study to screen these elite lines using *Pseudomonas* and A2C responses showed the clear potential to use in vitro systems, but these lines have to be further evaluated in greenhouse/field systems. Under in vitro conditions *Pseudomonas* and A2C treatments provide an insight about biomass- and metabolite-producing capabilities of specific clonal lines. In LH-14 it was clear that *Pseudomonas* stimulated metabolites toward the phenylpropanoid pathway with increase in biomass-producing ability and chlorophyll synthesis. Because A2C is an inhibitor of proline dehydrogenase (Elthon and Stewart, 1984) and proline is known to reverse A2C inhibition of differentiation in mammalian systems (Jost et al., 1988), the A2C tolerance of LH-14 suggested that proline may be a preferred source of reductant for energy (ATP synthesis) during response to A2C and *Pseudomonas*. Therefore, proline-linked respiration, in place of an NADH-linked one, may more efficiently generate energy as well as stimulate the pentose phosphate pathway to make available $NADPH_2$ and sugar phosphates for all anabolic needs (Figure 1; Phang, 1985; Shetty, 1997). Because chlorophyll was stimulated in response to both treatments, this suggested that cytokinins via purine metabolism may also be efficiently produced, because it is known that cytokinins can stimulate chlorophyll synthesis (Stoynova et al., 1996). Cytokinin may stimulate chlorophyll biosynthesis by affecting the amount of mRNAs related to chlorophyll *a*/*b* binding protein and the small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase (Cotton et al., 1990; Mok, 1994; Vitoria and Mazzafera, 1997; Rodoni et al., 1998). Biomass, RA, and phenolic stimulation may indicate that $NADPH_2$ and sugar phosphates were efficiently produced for overexpression of the phenylpropanoid pathway for the synthesis of free as well as condensed phenolics such as lignin on the cell wall. Lines LH-15 and LH-17, on the other hand, clearly indicate that *Pseudomonas* and A2C tolerance were accommodated by diversion of metabolites preferentially toward the phenylpropanoid pathway, and this diversion may be supported by the reduction of biomass and reduced energy for other anabolic pathways. Likewise, LH-11 is another interesting clonal line having a metabolic pathway that appeared to be more easily and preferentially diverted toward the phenylpropanoid pathway with possible compensation from other pathways including purine synthesis (because cytokinin-linked multiple shoot forming ability was reduced).

Because the goal of our research project was to produce high phenolic antioxidant-producing clonal lines, the above clonal lines will be ideal for further

greenhouse and field evaluation of metabolite profiles at the whole plant level. These lines will be compared to *Pseudomonas* and/or A2C susceptible clonal lines such as LH-3, LH-10, and LH-20. Phenolic antioxidant profiles of field-grown lines will be determined via HPLC, and in vitro antioxidant levels will be analyzed at various developmental stages of clonally regenerated plants. Line LH-5 will be also evaluated to determine whether A2C tolerance can also screen for overexpression of the terpenoid pathway, despite the in vitro phenolic pathway not being stimulated. This two-step *Pseudomonas* and A2C screening method provides a better logical strategy to regenerate clonal lines at the tissue culture level. These clonal lines have higher potential for large-scale clonal propagation (higher multiple shoot forming ability) and the method also concurrently isolates potentially high phenolic secondary metabolite-producing lines for greenhouse and field production. Clonal production of specific phenolic secondary metabolite profiles provides an opportunity to generate consistent phytochemicals for food, medicinal, and cosmetic applications of lavender.

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