Selection of High Phenolics-Containing Clones of Thyme (*Thymus vulgaris* L.) Using *Pseudomonas Sp.*

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A high phenolics-containing clonal line (T-12) of thyme (*Thymus vulgaris* L.) was isolated from a heterogeneous seed population by plant tissue culture techniques. This clonal line was isolated from among 10 clonal lines, with each originating from different genetically heterozygous, single-germinating seedlings. All clonal lines were generated via shoot organogenesis through adventitious bud proliferation from apex explants. Optimum shoot organogenesis was induced on Murashige and Skoog (MS) medium with benzyladenine (1 mg/L) as the growth hormone. Multiple shoots originating from apex explants of single heterozygous seedlings were further multiplied on the aforementioned benzyladenine-containing MS medium to subsequently generate a larger number of clonally identical plants. Shoots from each individual clonal line were inoculated with a novel *Pseudomonas* sp. Following growth on hormone-free MS medium for 25 days, total phenolics were determined spectrophotometrically. Using this approach, high phenolics-stimulated clonal line T-12 and moderate phenolics-stimulated clonal time T-16G were isolated. These clonal lines attained the higher level of phenolics following *Pseudomonas* inoculation and also had uninhibited shoot growth compared with the corresponding uninoculated control. Several low phenolics clonal lines, which had inhibited shoot growth in response to *Pseudomonas* sp., were also isolated. Thymol levels of uninoculated shoots of all clonal lines after 60 days of growth were also measured by gas chromatography−mass spectroscopy. The high-to-medium phenolics-containing clonal lines (T-12 and T-16G) had basal thymol levels in the range of 150 μg/g fresh weight (FW). The thymol levels of low phenolics-containing clonal lines were in the range 10−70 μg/g FW. This *Pseudomonas* sp.-mediated selection provides a potentially novel biotechnology based strategy to isolate high phenolics and thymol-containing clonal lines of thyme from a genetically heterogeneous population.

Keywords: *Biotechnology; clonal lines; micropropagation; phenolics; Pseudomonas sp.; thymol; Thymus vulgaris*

INTRODUCTION

Essential oils from thyme (*Thymus vulgaris* L.) have antioxidant properties, which may result from the presence of free radical scavengers in these oils (Farag et al., 1989; Deighton et al., 1993, 1994; Aeschbach et al., 1994). The antioxidant property of essential oils of thyme is specifically linked to the phenolic metabolites thymol and carvacrol (Aeshbach et al., 1994; Deighton et al., 1994; Madsen and Bartlesen, 1995). In addition, these compounds have been shown to have bactericidal and fungicidal properties (Beuchat, 1976; Conner and Beuchat, 1984; Karapmar and Aktug, 1987; Akgul and Kiranc, 1988; Sharpiro et al., 1994; Curtis et al., 1996). Therefore, these essential oils are good sources of natural preservatives for a variety of food and medical applications.

The current source of thyme essential oils containing thymol and carvacrol is from genetically heterogeneous plants. This genetic variation is similar to that of oregano (Fleischer and Sneer, 1982). The heterogeneity is due to the influence of natural cross-pollination on the breeding character, which is typical of species like thyme that belong to the family Lamiaceae. This heterogeneity results in a high degree of variability in the levels of active ingredients which poses serious problems for the routine use of thyme essential oils.

The current breeding and selection methods for thyme are not well developed. Therefore, variable essential oil extracts from plants have a high degree of heterogeneity even when the plants are from the same source or region of production. To maintain ingredient quantity and quality it is essential to develop elite plant varieties with uniform genetic backgrounds. This process may be long and difficult by traditional plant breeding techniques because of the poor knowledge of the genetic background of thyme and the high degree of heterozygosity. The strategy undertaken to overcome these problems is the basis of the research described in this manuscript. The strategy is based on the use of tissue culture-based clonal propagation of thyme and subsequent metabolic response of specific clonal lines to a unique *Pseudomonas* sp. The implications of such a selection strategy for further biochemical pathway analysis and commercial applications are also discussed.

MATERIALS AND METHODS

Tissue Culture of Thyme (*T. vulgaris* L.). Clonal lines of thyme were generated via adventitious shoot formation from individual heterozygous seedling following germination of a heterogeneous seed population. A cytokinin (benzyladenine)-induced adventitious shoot multiplication was used based on the method developed for melon (Shetty et al., 1992a) and soybean (Shetty et al., 1992b). Nodal segments of sterile

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Figure 1. Benzyladenine-induced multiple shoot formation from apex explants of thyme (magnification at 3.35× using Olympus-sz40 stereomicroscope).

seedlings (a bulk collection of seeds for germination were obtained from a heterogeneous seed lot sold by C. S. Hart Company, Chesterfield, CT) were dissected and cut in two along the axis from apex to base like with soybeans (Shetty et al., 1992b). Then dissected segments were transferred to benzyladenine (1 mg/L)-containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and 2 mM proline, like in melon organogenesis (Shetty et al., 1992a).

Adventitious shoots of each clonal line originating from a single heterozygous seed were induced from cut explants in 30−35 days (Figure 1). Each new shoot of every clonal line was used to generate more adventitious shoots, which were then used to generate larger numbers of clonally identical plants following rooting on half-strength (hormone-free) MS medium. With this approach, 100 clonal lines were developed and 10 were used in this study. Each clonal line originated from a different heterozygous seed. About 70% of the seeds from the heterogeneous seed lot were lost due to seed-borne infection that grow easily on a nutrient-rich medium. All tissue culture media and chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Inoculation with *Pseudomonas* sp. and Selection of High Phenolic-Producing Clones. A *Pseudomonas* sp. strain F was isolated during the tissue culture of oregano (Shetty et al., 1995). This *Pseudomonas* sp. prevented hyperhydricity linked problems associated with oregano tissue culture (Shetty et al., 1995). A consequence of preventing hyperhydricity in oregano was increased synthesis of phenolics (Shetty et al., 1995).

Individual shoots of thyme after clonal multiplication were inoculated with the *Pseudomonas* sp. strain F (Shetty et al., 1995). This *Pseudomonas* strain was grown on yeast extract-mannitol medium (DIFCO, Inc., Detroit, MI) until the inoculum density reached 10^9 colony-forming units/mL. The bacterial suspension (1 mL) was diluted with sterile distilled water (to 100 mL) and then dispensed into petri plates. Individual shoots of various clonal lines were then inoculated by dipping into the diluted bacterial suspension and then transferred to half-strength hormone-free MS medium (Shetty et al., 1995). Alternatively, inoculation was done by dipping shoots onto bacterial colonies grown on agar medium. The level of phenolics was measured 25 days after inoculation. Clonal lines that had high phenolics and normal growth 25 days after inoculation were characterized as elite lines. All uninoculated, high, medium, and low phenolics clonal lines were grown further for up to 60 days to allow differentiation of leaf glandular cells and analyzed for thymol content by gas chromatography.

Total Phenolics Assay. The phenolic assay was carried out with Folin−Ciocalteu reagent that was previously used for assaying phenolics in grapes (Singleton and Rossi, 1965) and modified for *Solanum* sp. (Chandler and Dodds, 1983) and soybean (Shetty, 1989). Leaf tissue (0.5 g FW) was transferred to 5 mL of 95% ethanol, disrupted with a Tissue Tearor (Biospec Instruments, Racine, WI), and centrifuged to remove the ethanol fraction. The ethanol extract was reacted with an equal quantity of Folin−Ciocalteu reagent (Sigma). After 5 min, 1 mL of 5% sodium carbonate was added to stabilize the color. Absorbance was read at 725 nm with a Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY). A standard curve was developed with gallic acid in 95% ethanol. Absorbance values were converted to mg total phenolics/g FW tissue. Each value reported in this study is an average of three replicate assays of three separate extracts.

Isolation of Leaf Oil. Approximately 3 g of fresh leaves were clipped from thyme plants from each clone with solvent-rinsed stainless steel scissors and tweezers. Leaves were transferred to a 500-mL distillation flask containing 250 mL of distilled deionized water. The flask was subsequently connected to a modified Likens−Nickerson steam codistillation apparatus (J&W Scientific, Folsom, CA). After extraction with *n*-pentane for 2 h, the pentane was recovered and concentrated to 1 mL under a stream of dry nitrogen. The extract was stored at −20 °C prior to analysis.

Gas Chromatography/Flame Ionization Detection (GC/FID) Analysis. Analyses were performed with a Hewlett-Packard 5890 gas chromatograph. The GC oven was fitted with 30 m × 0.32 mm DB-Wax fused silica capillary column (J&W Scientific). The liquid phase film thickness was 0.25 μm. The GC oven was temperature programmed as follows: 40 °C (hold 8 min), increase at 4 °C/min to 240 °C, hold 12 min. Helium carrier gas head pressure was fixed at 85 kPa, with injection at 250 °C in the splitless mode. Each value reported in this study is an average of two independent analysis from two separate extractions.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis. GC/MS analyses were performed on a Hewlett-Packard 5989 GC/MS system under chromatographic conditions identical to those described for the GC/FID analysis. The GC column was directly coupled to the ion source through a heated transfer line maintained at 280 °C. The mass spectrometer was operated in the electron-impact mode at 70 eV.

Reference Compounds. Reference standards of thymol and carvacrol were purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals used in this study were reagent grade.

RESULTS AND DISCUSSION

Tissue Culture of Thyme. Tissue culture-based propagation allowed isolation of clonal lines originating from heterozygous single seeds among a heterogeneous population. A cytokinin (benzyladenine)-induced adventitious shoot multiplication was used based on methods developed for melon (Shetty et al., 1992a) and soybean (Shetty et al., 1992b). About 7−10 adventitious shoots formed easily from each explant in 30−35 days (Figure 1). The supplementation of proline did not stimulate additional shoots but the quality of shoots in terms of morphological uniformity was enhanced (data

Table 1. Total Phenolic Levels, Thymol Content, and *Pseudomonas* Tolerance of Thyme Clones following Growth on Half-Strength Hormone-Free MS Medium[a]

clone	phenolics [mg/g FW (±SD)]	*Pseudomonas* tolerance[b]	thymol [μg/g FW (±SD)]
M-3 (control)	1.5 (0.2)	+	75 (10)
M-3 (inoculate)	2.0 (0.5)		
T-35G (control)	1.6 (0.4)	+	55 (10)
T-35G (inoculated)	2.2 (0.8)		
TM-4 (control)	1.9 (0.3)	+	80 (10)
TM-4 (inoculated)	2.2 (0.1)	+	
M-4 (control)	2.4 (0.2)	+	10 (5)
M-4 (inoculated)	2.2 (0.4)		
T-13G (control)	2.1 (0.2)	++	110 (20)
T-13G (inoculated)	2.6 (0.4)		
TM-2 (control)	2.5 (0.5)	+	110 (30)
TM-2 (inoculated)	3.0 (0.4)		
KM-40 (control0	1.3 (0.7)	++	125 (20)
KM-40 (inoculated)	3.2 (0.5)		
T-3 (control)	2.4 (0.5)	++	120 (15)
T-3 (inoculated)	3.3 (0.5)		
T-16G (control)	2.3 (0.2)	+++	150 (15)
T-16G (inoculated)	3.7 (0.6)		
T-12 (control)	2.7 (1.0)	++++	155 (15)
T-12 (inoculated)	4.8 (0.3)		

[a] *Pseudomonas* tolerance: (+) poor; (++) moderate-low; (+++) moderate-high; (++++) high.

not shown). Shoots were easily rooted on half-strength (hormone-free) MS medium to generate clonal plants originating from a single seed. Shoots were also used to generate more multiple shoots to enlarge each clonal line. This tissue culture approach to generate a clonal line originating from a single heterozygous seed among heterogeneous seeds is simple and does not require extensive hormone combinations described previously for thyme (Bajaj et al., 1988).

Selection of High Phenolics-Producing Clones. Following induction of multiple shoots from each clonal line, individual shoots were inoculated with a novel *Pseudomonas* sp. and transferred to half-strength MS (hormone-free) medium. Inoculated and uninoculated shoots of several clonal lines were analyzed for total phenolic content and tolerance to *Pseudomonas* sp. (Table 1). In this study, results of 10 clonal lines are presented. The basal phenolics content of all uninoculated clonal lines were in the range 2.0−2.5 mg/g FW. Inoculated shoots of clonal lines M-4, TM-4, TM-2, T-35G, and M-3 had marginal or no increase in total phenolics compared with uninoculated controls. Further, the growth of shoots was inhibited. Clones KM-40, T-16G, T-3, and T-13G had moderate increases in total phenolics, with partial inhibition of shoot growth. Only clonal line T-12 had a substantial increase in total phenolics coupled to normal growth and enhanced tolerance following *Pseudomonas* sp. inoculation. These results indicate that a clonal line that is tolerant to the *Pseudomonas* sp. is likely to contain high phenolics.

Essential Oil Analysis. The clonal lines containing different levels of phenolics were tested for thymol content by GC/FID (Table 1). Peak assignments were confirmed by GC/MS. Clonal line T-12 had the highest thymol content (155 μg/g FW). The moderate phenolic clonal lines T-16G, KM-40, T-3, and T-13G had thymol contents in the range 110−150 μg/g FW. The low phenolic clonal lines had thymol contents in the range 0−75 μg/g FW. These results indicate that high phenolics and *Pseudomonas* tolerance of clonal lines are excellent indicators of high thymol content.

Discussion and Implications. Genetic heterogeneity in thyme and other plants of the family Lamiaceae results in high variability in secondary metabolite content. This heterogeneity also poses problems for biochemical characterization of metabolite synthesis in thyme. Tissue culture-based propagation provides a means for generation of high thymol-containing clonal lines originating from a single seed among a heterogeneous and heterozygous population. This approach can be applied to any species that is open-pollinated (therefore very heterogeneous and heterozygous).

The selection of a high phenolic-containing clonal line with *Pseudomonas* sp. is a unique method to select high phenolic lines. The rationale for this selection is that the phenylpropanoid pathway is critical for plant response to microbial attack (Ebel, 1986; Dixon and Lamb, 1990). The novel *Pseudomonas* sp. was isolated during tissue culture of oregano (Shetty et al., 1995). This *Pseudomonas* sp. prevented hyperhydricity linked problems associated with oregano tissue culture (Shetty et al., 1995). A consequence of preventing hyperhydricity in oregano was increased synthesis of phenolics. Only clonal lines that produced high phenolics in response to *Pseudomonas* sp. survived and had reduced hyperhydricity. Oregano and thyme are closely related and belong to the family Lamiaceae. In this study this nonpathogenic bacterium allowed normal growth of thyme clonal line T-12 that had the capacity to produce high phenolics and high thymol content. The most significant observation was that the high thymol clonal line was not inhibited in growth in response to *Pseudomonas* sp. Growth of low phenolics clonal lines was inhibited. Therefore, there is a direct correlation between high phenolics, *Pseudomonas* tolerance, and growth of individual clonal lines.

Implications of this research are that with tissue culture-based clonal propagation coupled to *Pseudmonas*-mediated selection, high phenolics clonal lines can be developed from a heterogeneous population. This approach can then be extended to other species to multiply elite lines in large scale greenhouse production systems or elite clonal lines can be used in breeding programs to increase metabolite levels. Isolation of a clonal line giving rise to genetically uniform plants is also essential for characterization of specific biochemical pathways associated with phenolic metabolites.

These results are promising, but further work is also needed to determine if tissue culture-based elite clonal lines continue to produce high levels of phenolics at various developmental stages and when growing in conventional ex vitro production systems. We are now acclimatizing tissue culture-generated, rooted plants for greenhouse evaluation of thymol content in high phenolic clonal line T-12 and low phenolic clonal line M-3. Thymol analysis will be done at this later developmental stages when glandular cells for thymol accumulation are well developed in the leaves.

Conclusions. With increasing importance of thymol-containing thyme extracts in food and pharmaceutical application (see Introduction), our results offer a novel biotechnological strategy to select elite thymol-containing clonal lines. Tissue culture approaches can be adopted to isolate genetically uniform clonal plants. Using *Pseudomonas* sp., high phenolics clonal lines can

be isolated and further propagated for various food and industrial applications. In addition, the genetic uniformity of clonal plants from a single line will allow study of biochemical pathways associated with thymol synthesis. It will also be interesting to study whether high thymol-containing clonal lines offer higher resistance to microbial attack and whether thymol levels are stimulated like an elicitor response.

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LITERATURE CITED

Aeschbach, R.; Loliger, J.; Scott, B. C.; Murcia, A.; Butler, J.; Haliwell, B.; Aruoma, O. I. Antioxidant actions of thymol, carvacrol 6-gingerol, zingerone and hydroxytyrosol. *Food Chem. Toxicol.* **1994**, *32*, 31−36.

Akgul, A.; Kivanc, M. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. *Int. J. Food Microbiol.* **1988**, *6*, 363−368.

Bajaj, Y. P. S.; Farmanowa, M.; Olszowska, O. Biotechnology of the micropropagation of medicinal and aromatic plants. In *Biotechnology in Agriculture and Forestry*; Bajaj, Y. P. S., Ed.; Springer-Verlag: Berlin, 1988; pp 60−103.

Beuchat, L. R. Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. *J. Food Sci.* **1976**, *41*, 899−902.

Chandler, S. F.; Dodds, J. H. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum Laciniatum*. *Plant Cell Rep.* **1983**, *2*, 105−108.

Conner, D. E.; Beuchat, L. R. Effects of essential oils from plants on growth of food spoilage yeast. *J. Food Sci.* **1984**, *49*, 429−434.

Curtis, O. F.; Shetty, K.; Cassagnol, G.; Peleg, M. Comparisons of the inhibitory and lethal 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.

Deighton, N.; Glidewell, S. M.; Deans, S. G.; Goodman, B. A. Identification by EPR spectroscopy of carvacrol and thymol as the major sources of free radicals in the oxidation of plant essential oisl. *J. Sci. Food Agric.* **1993**, *63*, 221−225.

Deighton, N.; Glidewell, S. M.; Goodman, B. A.; Deans, S. G. The chemical fate of the endogenous plant antioxidants carvacrol and thymol during oxidative stress. *Proc. R. Soc. (Edinburgh)* **1994**, *102B*, 247−252.

Dixon, R. A.; Lamb, C. J. Molecular Communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 339−367.

Ebel, J. Phytoalexin synthesis: the biochemical analysis of the induction process. *Annu. Rev. Phytopathol.* **1986**, *24*, 235−264.

Farag, R. S.; Badei, A. Z. M. A.; ElBaroty, G. S. A. Influence of thyme and clove essential oils on cottonseed oil oxidation. *J. Am. Oil Chem. Soc.* **1989**, *66*, 800−804.

Fleisher, A.; Sneer, N. Oregano spices and *Origanum* chemotypes. *J. Sci. Food Agric.* **1982**, *33*, 441−446.

Karapmar, M.; Aktug, S. E. Inhibition of food borne pathogens by thymol, eugenol, menthol and anethole. *Int. J. Food Microbiol.* **1987**, *4*, 161−166.

Madsen, H. L.; Bertelsen, G. Spices as antioxidants. *Trends Food Sci. Technol.* **1995**, *6*, 271−277.

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

Shapiro, S.; Meier, A.; King, J. The antimicrobial activity of essential oils and essential oil components towards oral anaerobes. *Oral Microbiol. Immunol.* **1994**, *9*, 202−208.

Shetty, K. Microbial elicitors and secondary metabolite production in plant cell suspension cultures, phenolics as a model system. Ph.D. Thesis, University of Idaho, **1989**.

Shetty, K.; Asano, Y.; Oosawa, K. Stimulation of *in vitro* shoot organogenesis in *Glycine max* (Merill) by allantoin and amides. *Plant Sci.* **1992a**, *81*, 245−251.

Shetty, K.; Shetty, G. A.; Nakazaki, Y.; Yoshioka, K.; Asano, Y.; Oosawa, K. Stimulation of benzyladenine-reduced *in vitro* shoot organogenesis in *Cucumis melo* by proline, salicyclic acid and aspirin. *Plant. Sci.* **1992b**, *84*, 193−199.

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* sp. *Plant Physiol.* **1995**, *147*, 447−451.

Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with physphomolybdic-phosphotungstic acid. *Am. J. Enol. Vitic.* **1965**, *163*, 144−158.

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