Effect of Selected Polysaccharide-Producing Soil Bacteria on Hyperhydricity Control in Oregano Tissue Cultures

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Hyperhydricity, or vitrification, is a physiological malformation affecting tissue culture-generated plants. This malformation is associated with excessive hydration and poor lignification and results in poor regeneration of plants. We have tested hyperhydricity prevention in oregano by several nonspecific polysaccharide-producing rhizosphere bacteria. Among these bacteria, *Pseudomonas mucidolens* and another *Pseudomonas* sp. prevented hyperhydricity and improved acclimation of oregano clones. These two bacteria have more advantages for commercial applications than *Pseudomonas* strains isolated previously from oregano.

Hyperhydricity, or vitrification, is a physiological malformation affecting clonally propagated plants generated under tissue culture conditions (6, 9). Hyperhydrated plants are enlarged, thick, translucent, and brittle. This physiological malformation is associated with chlorophyll deficiency, poor lignification, and excessive hydration of tissues (9), which result in poor regeneration of normal, mature plants (2, 11, 13, 14, 17). Several empirical approaches have been tested to reduce hyperhydricity in tissue culture in order to improve the efficiency and quality of clonal propagation. These approaches include increasing carbohydrate levels in the medium (12, 17), changing light intensity (16), modifying concentrations of gelling agents (1, 5, 17), reducing humidity (2), and using Bacto Peptone and its subfractions (13), but successes have been limited.

Recently, a unique polysaccharide-producing *Pseudomonas* sp. that prevented hyperhydricity during in vitro shoot culture of oregano (*Origanum vulgare*) was isolated (14, 15). This *Pseudomonas* sp. prevented hyperhydricity in several clones of oregano after 10 to 15 days of inoculation. The inoculated shoots were normal, with lower water content and higher levels of chlorophyll and phenolics than hyperhydrated shoots. The efficiency of regeneration of *Pseudomonas* sp.-mediated, hyperhydricity-negative plants was >90% for the clones tested. This novel approach allowed rapid clonal propagation of a high percentage of plants from an elite clone; the clones were well acclimated for growth in the outside environment or in a greenhouse following tissue culture-based clonal multiplication.

Based on the earlier studies with this *Pseudomonas* sp., we have evaluated the effects of selected plant- and rhizosphere-associated bacteria on prevention of hyperhydricity in oregano clones. This work resulted in the screening of two polysaccharide-producing nonspecific bacteria, *Pseudomonas mucidolens* and a *Pseudomonas* sp., that prevented hyperhydricity in oregano. These nonspecific bacteria are potential targets for commercial applications in the plant tissue culture industry.

The following bacterial strains were obtained from the American Type Culture Collection, Rockville, Md.: *P. mucidolens* ATCC 4685, *Pseudomonas* sp. strain ATCC 31461, *Pseu-*

domonas stutzeri ATCC 31258, and *Beijerinckia indica* ATCC 21423 (see Table 1). All strains of *Pseudomonas* used in this study produce extracellular polysaccharide and were originally isolated from soil. *B. indica* is a plant-associated soil bacterium that produces a heteropolysaccharide. All *Pseudomonas* strains were grown in nutrient agar or yeast extract-mannitol agar (YMA) at 25°C, and *B. indica* was grown in YMA at 25°C for 48 to 60 h. Following growth of bacteria, plates were stored at 5°C. The polysaccharide-producing *Pseudomonas* sp. strain F, which prevented hyperhydricity in oregano (14), was used as a positive control.

Shoot cultures from a clonal line of oregano (clone O-1) were generated previously (14) and were used in this study to compare the effects of various bacteria. The shoots were subcultured on Murashige and Skoog (MS) medium (10) containing 1 mg of benzylaminopurine per liter, 3% sucrose, and 0.3% gellan gum (Sigma Chemical, St. Louis, Mo.). The medium was adjusted to pH 5.8 before being autoclaved at 121°C for 15 min. Each petri plate contained seven apices, and each apex had four lateral leaves below it. These petri dishes were incubated at 20°C under continuous light of 40 $\mu\text{mol}/\text{m}^2/\text{s}$. After 30 days, the shoot apex explants had regenerated several more apices through axillary bud proliferation. The O-1 clonal line was maintained by subculturing the shoots at 30-day intervals. Shoots of new apices of oregano clone O-1 cultured for 30 days were inoculated by gently treating the stem end with various bacteria previously stored at 5°C on YMA or nutrient agar plates. The treated shoot apex explants were transferred to half-strength MS medium containing 1.5% sucrose. Each treatment had at least four petri plates (replicates) with seven explants in each petri plate. After 30 days of shoot growth and multiplication, hyperhydricity-related physiological parameters were measured.

Hyperhydrated and bacterially mediated unhyperhydrated shoots were compared with respect to total phenolics, chlorophyll content, water content, and fresh weight. Results are averages of three samples with standard deviations. Total phenolics were determined after extraction from 50 mg (fresh weight) of shoot placed in 2.5 ml of 95% ethanol, and determination was based on previous methods (3, 14). The extracted phenolics were reacted with Folin-Ciocalteu reagent followed by 5% Na_2CO_3 , and absorbances were read at 725 nm with 95% ethanol as a blank, by using a Genesys spectrophotometer (Spectronic, Inc., Rochester, N.Y.). Standard curves were es-

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TABLE 1. Average water content in control and bacterially inoculated oregano shoot explants from clonal line O-1 30 days after treatment

Treatment	Total chlorophyll ($\mu\text{g/g}$ [fresh wt] of tissue) (mean $\pm$ SD)	mg of water in 100 mg of tissue (mean $\pm$ SD)	Phenolics		Plant growth (biomass) (mg/replicate)
			mg/g (fresh wt) of tissue (mean $\pm$ SD)	$\mu\text{g/g}$ (dry wt) of tissue (mean $\pm$ SD)	
None (control)	698 $\pm$ 56	91.2 $\pm$ 0.9	1.8 $\pm$ 0.1	20.7 $\pm$ 1.3	465
<i>P. mucidolens</i>	1,102 $\pm$ 42	89.2 $\pm$ 0.4	2.8 $\pm$ 0.2	26.2 $\pm$ 1.9	243
<i>Pseudomonas</i> sp. strain ATCC 31461	1,040 $\pm$ 59	88.5 $\pm$ 0.8	2.6 $\pm$ 0.2	22.2 $\pm$ 1.3	264
<i>P. stutzeri</i>	888 $\pm$ 155	84.2 $\pm$ 0.8	2.8 $\pm$ 0.2	17.7 $\pm$ 1.3	159
<i>B. indica</i>	906 $\pm$ 41	85.3 $\pm$ 0.7	4.6 $\pm$ 0.2	31.5 $\pm$ 1.1	99
<i>Pseudomonas</i> sp. strain F	1,048 $\pm$ 35	85.9 $\pm$ 1.0	4.8 $\pm$ 0.5	33.8 $\pm$ 3.7	130

tablished for each experiment with various concentrations of gallic acid in 95% ethanol. Absorbance values were converted to milligrams of phenolics per gram (fresh weight) of tissue. The values were also expressed on a dry weight basis. Total chlorophyll was estimated by the spectrophotometric method after extraction in methanol (8). The absorbance was measured at 650 and 665 nm. The chlorophyll concentration was expressed in micrograms per milliliter of methanol and was calculated as follows: total chlorophyll = $25.8 \times A_{650} + 4.0 \times A_{665}$, where A_{650} and A_{665} are absorbances at 650 and 665 nm, respectively. The chlorophyll levels were then converted to micrograms of chlorophyll per gram (fresh weight) of tissue by the formula [(micrograms of chlorophyll/milliliter of methanol) $\times$ 3 ml of methanol]/(gram of tissue). Water content was measured by placing 150 mg (fresh weight) of explants in preweighed pans, drying them for 2 h at 105°C, and subsequently cooling them for 30 min in a desiccator. Pans were weighed after 1.5 h to confirm weight constancy. Water content was estimated and expressed on a 100-mg fresh weight basis by the formula [(fresh weight - dry weight)/(fresh weight)] $\times$ 100.

Plant growth (biomass) was measured as the total fresh weight of five replicates divided by the number of replicates.

The ability of shoots to acclimate outside the tissue culture environment was tested. Thirty days after inoculation, both hyperhydrated and bacterially mediated unhyperhydrated shoots were subcultured for rooting on hormone-free half-strength MS medium. After 30 days, these plants were transferred to vermiculite and covered with transparent plastic to provide high humidity (100%). After 5 days, the plastic cover was replaced by another cover with perforations for 2 days. Then the cover was completely removed. The exposed plants were placed under a regimen of 16 h of light per day at 25°C and 80% humidity in a room with conditions similar to a greenhouse.

After 1 or 3 weeks of acclimation the number of plants that survived the change in humidity was determined. In addition, the vigor of each plant was scored visually on a five-point scale (–, $\pm$, +, ++, and +++).

Inoculated shoots were dark green, opaque, and rigid, whereas control shoots were, brittle, elongated, glassy, and light green, indicating low chlorophyll levels consistent with hyperhydricity (9, 14). All bacterial treatments had reduced the level of water compared to the control (Table 1). Among the treatments, *P. mucidolens* and *Pseudomonas* sp. strain ATCC 31461 inoculations caused moderate reductions in water content compared to inoculation with *P. stutzeri*, *B. indica*, or *Pseudomonas* sp. strain F (isolated from oregano). This reduced water content was required for enhanced acclimation of unhyperhydrated plants (9, 14). Excessive hydration is one characteristic associated with hyperhydrated tissue; it may be related to lower levels of lignin synthesis in the xylem vessels

and may result in abnormal and diffused water conduction and therefore in brittleness.

The results in Table 1 indicate that in oregano shoots inoculated with *P. mucidolens*, *Pseudomonas* sp. strain ATCC 31461, and *P. stutzeri*, phenolics were moderately stimulated when expressed on a fresh weight basis, but the level of stimulation was lower than our previously recorded level for *Pseudomonas* sp. strain F inoculation (14). However in the present study, *Pseudomonas* sp. strain F- and *B. indica*-inoculated plants had much higher levels of phenolics at day 30 than the other plants, similar to results from a previous study (15). On a dry weight basis, *P. mucidolens*, *Pseudomonas* sp. strain F, and *B. indica* induced significantly higher levels of phenolics than *Pseudomonas* sp. strain ATCC 31461 and *P. stutzeri* (Table 1). Overall, the results agree with previous observations showing that phenolics in oregano are stimulated under reduced-hyperhydricity conditions (9, 15). The increase in phenolic production linked to various polysaccharide-producing bacteria may be similar to elicitor-mediated stimulation of secondary metabolite synthesis in plants (4, 7). This may be the mode of regulation of phenolic metabolite flux required for lignin synthesis in unhyperhydrated tissues (9).

Chlorophyll levels in bacterially inoculated shoots were much higher than those in uninoculated shoots, indicating normal photosynthesis. The chlorophyll levels in *P. mucidolens*-, *Pseudomonas* sp. strain ATCC 31461-, and *Pseudomonas* sp. strain F-inoculated shoots were higher than those in *P. stutzeri*- and *B. indica*-inoculated shoots (Table 1). The higher chlorophyll levels may directly correlate with high photosynthetic efficiency, contributing to enhanced growth, increased stress tolerance, and better acclimation (Table 2). In the case of uninoculated control shoots, the lower chlorophyll content and higher water content may cause a physiological imbalance

TABLE 2. Evaluation of normal development and acclimation in soil of control and bacterially inoculated oregano shoot explants of line O-1 60 days after treatment

Treatment	No. of explants	% of shoots hyperhydrated	% Survival after acclimation for:		Plant vigor after 3 wk of acclimation ^a
			1 wk	3 wk	
None (control)	15	93	100	73	+
<i>P. mucidolens</i>	15	0	100	93	+++
<i>Pseudomonas</i> sp. strain ATCC 31461	15	0	100	93	++
<i>P. stutzeri</i>	15	46	100	93	++
<i>B. indica</i>	7	0	100	100	+
<i>Pseudomonas</i> sp. strain F	7	7	100	86	++

^a +, low vigor; ++, medium vigor; +++, high vigor.

FIG. 1. Survival of oregano clone O-1 after 3 weeks of acclimation following treatment with various bacterial species to reduce hyperhydricity. Control, uninoculated control; a, *P. mucidolens*; b, *Pseudomonas* sp. strain ATCC 31461; c, *P. stutzeri*; d, *B. indica*; F, *Pseudomonas* sp. strain F. After bacterial treatment, the clones were enclosed with a transparent plastic cover for 1 week before sudden exposure to room temperature and humidity conditions.

leading to higher levels of hormones like indoleacetic acid, which could lead to the excessive elongation that was observed. This may reduce metabolic flux towards phenolics and lignin synthesis, thus weakening the stem and reducing the water-holding capacity of the xylem tissues. In previous studies, lower chlorophyll levels have been associated with hyperhydrated tissue (2, 9, 14). These negative attributes were reversed in all bacterially inoculated shoots in this study.

Polysaccharide-producing bacteria like *Pseudomonas* sp. strain F (15) reduced plant growth while reducing hyperhydricity. However, the reduction in plant growth was much lower in the case of *P. mucidolens* and *Pseudomonas* sp. strain ATCC 31461 inoculations (Table 1). In spite of the growth reduction with any bacterial inoculation, the proportions of new explants with the ability to form multiple shoots were similar to that of the uninoculated control due to a reduction in internode size. This results in an equal number of terminal or axillary buds for the next subculture to generate more clonal shoots. This indicates that the use of polysaccharide-producing *P. mucidolens* or *Pseudomonas* sp. strain ATCC 31461 was as effective as *Pseudomonas* sp. strain F inoculation in the ability to generate sufficient explants and prevent hyperhydricity. In many situations, *P. mucidolens* and *Pseudomonas* sp. strain ATCC 31461 could be better because the shoot growth and internode size were not reduced in comparison to those obtained with *Pseudomonas* sp. strain F inoculation, facilitating easy culturing of explants. Therefore, perhaps *P. mucidolens* and *Pseudomonas* sp. strain ATCC 31461 can be effectively targeted for commercial development to optimally reduce hyperhydricity in the commercial tissue culture and micropropagation industry.

Acclimation studies (Table 2) indicate that *P. mucidolens*, *Pseudomonas* sp. strain ATCC 31461, and *Pseudomonas* sp. strain F cause the greatest reductions in hyperhydricity and enhancements of vigor of plants. Inoculations with these bacteria result in easy acclimation to outside environmental conditions without much conditioning in the greenhouse-like con-

ditions that are normally required for several tissue-cultured plant species (Fig. 1). These results also could imply that plant acclimation to the environment has a close relationship to compatible bacteria. Therefore, plants in tissue culture conditions may require such beneficial bacteria for efficient acclimation to the outside environment, and without these bacteria the plants have reduced vigor and stress tolerance.

Although various empirical strategies have been tested to reduce hyperhydricity, no strategy is as effective as bacterial inoculations. The bacterial species tested in this study offer opportunities to overcome some of the problems associated with growth reduction with extremely high inocula, as observed with *Pseudomonas* sp. strain F (14). A high inoculum density of *P. mucidolens* or *Pseudomonas* sp. strain ATCC 31461 reduced growth by only 40 to 50%. This reduction may be the cost of the stress and adaptive response, and it did not result in a decrease in the number of explants that were generated for the next cycle of propagation, due to similar numbers of internodes. Therefore, the use of nonspecific polysaccharide-producing bacteria offers excellent opportunities to solve hyperhydricity problems. Most importantly, this oregano-bacterium interaction offers a good system for understanding the mechanisms underlying hyperhydricity. Our immediate goal is to use *P. mucidolens* and *Pseudomonas* sp. strains ATCC 31461 and F in commercial tissue cultures, like raspberry cultures, where hyperhydricity is a serious problem. Further, we are investigating whether polysaccharide-negative mutants of *P. mucidolens* and *Pseudomonas* sp. strain ATCC 31461 can reduce hyperhydricity. The results should indicate the importance of polysaccharide in hyperhydricity reduction, as in studies with *Pseudomonas* sp. strain F (14, 15).

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