Enhancing the regeneration efficiency of lavandin (Lavandula x intermedia cv Grosso): effects of light quality, medium strength, phenolic control agents, and polyamines

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Abstract
An efficient protocol for the regeneration of lavandin (Lavandula x intermedia cv Grosso) is reported. Thiadiazuron (9 μM), a plant growth modulating phenylurea, was used to induce callus formation and shoot initiation from cultured leaf explants. Newly emerged shoots were maintained on media containing 0.05 μM naphthaleneacetic acid to allow maturation, and then transferred to media containing 2.9 μM indole-3-acetic acid to allow root formation. The phenolic control agents polyvinylpyrrolidone, ascorbic acid, 2-aminoindane-2-phosphonic acid and activated charcoal were tested for their ability to prevent shoot browning and death in culture. All agents except PVP were found to be effective, with ascorbic acid being most consistent in promoting development of healthy mature shoots. The effect of light type (red light vs. white light) and culture medium composition (full and half strength Murashige and Skoog medium, and Lloyd and McCown’s woody plant medium (WPM)) on rooting efficiency was also evaluated. Cultures on half strength WPM in white light were found to have the highest rooting efficiency. Additionally application of the polyamines putrescine, spermine and spermidine were tested for their effect on rooting. While rooting efficiency was not improved with any of the treatments, spermine and spermidine were found to have an inhibitory effect at concentrations greater than 10 μM.

Abbreviations

AA ascorbic acid,
AC activated charcoal
AIP 2-aminoindane-2-phosphonic acid
ANOVA analysis of variance
HSD honestly significant difference
IAA indole-3-acetic acid
MS Murashige and Skoog medium
Introduction

Lavenders (*Lavandula*) are a diverse group of aromatic plants in the *Lamiaceae* family grown worldwide both commercially and non-commercially as essential oil crops and as ornamentals (Upson and Andrews 2004a). It has been estimated that over 2000 tons of essential oil is produced annually from these plants, making them commercially valuable commercial crops. Mainly three species of lavender are grown commercially for their essential oil: English lavender (*L. angustifolia*), spike lavender (*L. latifolia*) and their hybrid, lavandin (*L. x intermedia*). *L. x intermedia* cv Grosso is the most widely grown commercial cultivar due to its significantly higher oil yield as compared to other lavender species such as *L. angustifolia* (English lavender) (Upson and Andrews 2004a). As a sterile hybrid, *L. x intermedia* cannot be propagated through seed, and therefore the establishment of tissue culture and micropropagation methods is necessary (Upson and Andrews 2004b). While micropropagation methods are most useful in the nursery, tissue culture methods allow for the application of biotechnology to introduce improved traits. They also allow for establishment of cultures that do not deteriorate in quality through multiple propagation steps, and for the production of individuals with improved traits such as higher oil yield, enhanced oil composition and increased vigor.

Browning is a major source of loss in the culture of plants, and is due in part to the accumulation of phenolics in the media. To date, three main classes of agents have been used to control browning of cultured plants. These include adsorbents such as activated charcoal (AC) and polyvinylpyrrolidone (PVP), which adsorb phenolics secreted into the media by cultured explants, reducing agents such as ascorbic acid, which chemically reduce the phenolics in the media (Katterman and Williams 1977; Pan and Staden 1998; Zuzarte et al. 2010), and phenolic inhibitors, which act directly on the plant to prevent secretion of phenolics in the first place. In this study the powerful and reversible phenylammonia lyase (PAL) inhibitor 2-aminoindane-2-phosphonic acid (AIP) was used at a low concentration (Jones et al. 2012; Zoń et al. 2005) to reduce production of excess phenolic compounds.

Media composition and light conditions are important determinants of regeneration efficiency. In particular, the micro- and macronutrient components of media have a significant effect on plant regeneration. Specific formulations of these nutrients must often be developed for certain plant species to suite their particular nutritional requirements. Although most cultures are maintained under white light, the successful use of red light has been reported in other woody plant species (Daud et al. 2013). All previously reported lavender regeneration procedures have used
white light for rooting, and the effects of red light on rooting efficiency in lavenders have not been examined.

Rooting efficiency can also be affected by polyamines, a small group of phytohormones comprised of the triamine spermidine (Spd), the tetraamine spermine (Spm) and their precursor putrescine (Put), a diamine. These compounds have been reported to improve plant response to stressful conditions such as high salinity, and drought, as well as regulating plant growth and development (Gill and Tuteja 2010; Kusano et al. 2007). Exogenous application of polyamines was reported to improve rooting efficiency in some plant species, however, the effect of these compounds in lavender has not yet been tested (Couée et al. 2004).

To date, a protocol for the regeneration of L. x intermedia cv Grosso from leaf tissue has not been reported. In addition, the effects of various culture conditions on regeneration efficiency for this economically important plant have not been examined. Here we report an efficient protocol for the regeneration of L. x intermedia cv Grosso from leaf tissue. We also examined the impact of phenolic build-up inhibitors, and evaluated the effects of light conditions, media type, media composition, and polyamines on regeneration of these plants.

Materials and Methods

Mature Lavandula x intermedia var Grosso plants were obtained from Okanagan Lavender Herb Farm, Kelowna, BC, Canada. Leaf or node cuttings were removed and surface sterilized by immersion in 20% sodium hypochlorite with 0.1% Triton-X100 (Fisher Scientific, Canada) for 20 minutes with stirring. Explants were then washed three times with sterile water for 5 minutes each. To propagate shoots for shoot survival trials a previously reported method, used for propagating L. angustifolia was used with minor modifications. Briefly leaf explants were lacerated along the edges then plated abaxial side down on Murashige and Skoog (MS) media including vitamins (Murashige and Skoog 1962) (PhytoTechnology Laboratories, USA) containing 3% (w/v) sucrose (Fisher Scientific, Canada), 2.35 % (w/v) gellan gum (PhytoTechnology Laboratories, USA) and 9 μM thiadazuron (TDZ) (PhytoTechnology Laboratories, USA), pH was adjusted to 5.8 prior to autoclaving. Cultures were kept in the dark at 25°C for approximately three weeks until shoot production was observed (Falk et al. 2009). Shoots were then excised from the callus and moved to a 16-hour photoperiod and allowed to multiply. Newly emerged shoots were sub-cultured every 14 days for a month to establish sufficient number of shoots. These shoots (n=12 for each treatment group) were then placed one per culture box on MS medium (as described above) containing 0.05 μM naphthaleneacetic acid (NAA) and one of each of the following treatments: 10 mg/L L-ascorbic acid (AA) (Acros Organics, Canada), 250 g/mL PVP (Sigma-Aldrich, Canada), 0.21 mg/L AIP (sv Chembiotech Inc. Edmonton, Canada), 0.5 g/L AC (EMD Millipore, USA), 1 g/L AC or no treatment. AA and PVP were filter-sterilized (0.22 μm, EMD Millipore, USA) and added after autoclaving, while AIP and AC were added prior to autoclaving. Cultures were kept at 25°C, 16-hour photoperiod for 4 weeks and browning and leaf morphology changes recorded. At the end of four weeks, shoot vigor was recorded...
on a scale of zero to four with categories defined as follows: 0 – brown and dead; 1 – brown, immature; 2 – brown, mature; 3 – green, immature; 4 – green, mature.

For rooting trials node cuttings were placed on either MS media or woody plant medium (WPM) (Lloyd and McCown 1980)(PhytoTechnology Laboratories, USA) at full or half strength with 3% (w/v) sucrose, 2.5% (w/v) gellan gum and 2.9 μM indoleacetic acid (Sigma-Aldrich, Canada) with pH adjusted to 5.8. Nodes cultured on each of the four media types were then kept on a 16-hour photoperiod under either white fluorescent or red LED light. Cultures were maintained for 4 weeks and root formation was recorded. For each treatment group n = 4, experiments were performed in triplicate.

The effect of the polyamines Put (PhytoTechnology Laboratories, USA), Spm (Sigma-Aldrich, Canada) and Spd (PhytoTechnology Laboratories, USA) on rooting efficiency was examined on plants that were grown on half-strength MS or half-strength WPM media in white light as described above. Polyamines were filter sterilized and added to the medium after autoclaving to obtain final concentrations of 10, 100 or 1000 μM. Cultures were maintained for 4 weeks and root formation was recorded. For each treatment n = 6, experiments were performed in triplicate.

Statistical analysis was carried out by analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) multiple comparisons model using R with α = 0.05. Contingency analysis was performed on shooting data using JMP v. 4.0. In all cases a full factorial design was used, with treatments randomly assigned to cultures.

Results

Organogenesis from leaf callus

A procedure previously developed for the regeneration of L. angustifolia plants (Falk et al. 2009) was used to regenerate Grosso plants from leaf segments. Noticeable callus was observed at injury sites on leaf tissue within two weeks of culture start date, and after three weeks calli readily produced viable shoots Approximately 5-10 shoots were produced per callus, which could then be excised and sub-cultured on MS media containing 9 μM TDZ (Figure 1). Shoots were transferred to media containing NAA to allow further development, and then to media containing IAA to promote rooting, however, most shoots did not survive this process due to browning and none of those that survived produced viable roots (Table 1).

Addition of phenolic inhibitors, adsorbents and reducing agents

Phenolic compounds secreted by cultured plants are a well-known cause of culture browning so in an attempt to reduce this phenomenon, the phenolic control agents AA, AC, AIP and PVP were added to the culture media. On a ranking scale where a dead explant was assigned a score of zero and a healthy mature shoot scored a four (Figure 2), the control group ranked at 0.42; treatment with PVP was not significantly better. Ascorbic acid, AC and AIP treatments all showed a significant
improvement in shoot vigor, with AA being the highest at 3.67. All additives except
PVP showed a significant improvement in shoot vigor as compared to the control,
although AA was the most effective agent with very little variability in results (Table
1). Both the control and PVP treatment also showed very low variability, however,
AC and AIP treatments were less consistent with large variability (Table 1). Only
42% of cultures in the control survived, and those that did were of poor quality. In
comparison 83% of all cultures started on AA treatment were of good quality and
reached maturity with 100% surviving the culture process. In comparison, AC (0.5
g/L and 1 g/L) and AIP treatments had 92, 75 and 92% survival, respectively, with
42, 50 and 50% being of good quality and reaching maturity, respectively. Only 50%
of shoots treated with PVP survived, with none reaching maturity (Figure 3).

Effect of light and media conditions

To determine the effects of light quality and medium strength, nodal cuttings from
Grosso plants were grown on full or half strength MS or WPM media under white
fluorescent or red LED light. Interaction plots were constructed for all treatment
combinations before ANOVA analysis was performed (data not shown) to determine
if significant interactions were occurring. Although no interaction was seen with
media type, a significant interaction was seen between light type and media
strength, with red light having a significant effect only on cultures on full strength
medium (p = 0.01). Media type had the strongest effect on rooting efficiency with
significantly more shoots grown on WPM showing rooting (P < 0.001), light quality
had a minimal effect (p = 0.01). A multiple comparisons model was performed on all
treatment combinations and found the effects of light to be diminished, with
treatment groups between red and white light often not showing any significant
difference (Figure 4). Although cultures under red light on full or half strength WPM
had the best rooting efficiency, these treatments were not significantly better than
cultures (at either strength) on WPM or half strength MS under white light. Red light
treatments under WPM did have higher rooting efficiency than white light
treatments, however, cultures under red light showed some etiolation, and
increased callus production, while explants under white light showed no etiolation
and generally had less callus production and more robust roots (Figure 5).

Effect of polyamines

Putrescine treatment did not have a significant effect on rooting efficiency at any
level (p = 0.35) while spermine (p <0.001) and spermidine (P < 0.001) treatments
showed significant inhibition of rooting. No significant interactions were found
between media type and polyamine treatment (data not shown). Both spermine and
spermidine showed significant rooting inhibition on both WPM and MS at
concentrations greater than 10 μM with 50% inhibition at 100 μM on MS for both
Spd and Spm, and 38% inhibition on WPM for Spd and 36% for Spm. Complete
inhibition occurred for Spm at 1 mM on both media types while the effect of Spd was
slightly weaker, with 83% inhibition occurring on both WPM and MS media.
Multiple comparison’s analysis found a significant difference in rooting efficiency at
the 10 μM level for spermine treatment on WPM, and a significant difference at the 1
mM level for spermidine treatments, and spermine on MS media (Figure 6).
Furthermore, explants on higher polyamine levels showed significant browning and tissue death while explants at lower concentrations were of higher quality (Figure 7).

Discussion

A method for the regeneration of *L. angustifolia* plants from leaf tissue was recently reported (Falk et al. 2009). Although callus production and shoot initiation was highly successful from Grosso leaf tissue (with up to 15 shoots per explant), shoot maturation and root induction were problematic as most shoots did not survive due to browning and none of those that did produced roots. After shoot initiation, shoot elongation and maturation must occur before shoots are able to produce viable roots. A major obstacle in this process, browning, can occur while shoots are still in an immature state (Figure 2). This browning is caused by phenolic compounds, produced by the cultured tissue, that build-up in the medium during the regeneration process and lead to tissue browning, which in many cases is lethal to the explant (Laukkanen et al. 1999; Tang and Newton 2004). Several classes of additives were used in this study to reduce browning and improve regeneration efficiency of Grosso plants. Polyvinylpyrrolidone and AC adsorb free phenolics in culture medium to decrease the phenolic compounds that are in contact with the explant. Ascorbic acid accomplishes the same by acting as a reducing agent whose use has previously been reported in *Lavandula pedunculata*. 2-Aminoindane-2-phosphonic acid is a relatively novel PAL inhibitor whose use has to date only been reported in St. John’s wort, duckweed, bay willow and elm; AIP inhibits phenolic production to prevent accumulation (Gitz et al. 2004; Hu et al. 2011; Jones et al. 2012; Klejdus et al. 2013; Pan and Staden 1998; Ruuhola and Julkunen-Titto 2003; Zuzarte et al. 2010). Shoots not treated with a phenolic control agent were of poor quality with few surviving. Treatment with AC, AIP or AA substantially improved overall shoot quality and survival (Table 1), with AA having the least variable results indicating that it is a preferable treatment. Some of the variability seen in AC and AIP treatments could be related to an inhibition of growth by these compounds, a phenomenon that has been reported for certain plant species (Klejdus et al. 2013; Pan and Staden 1998). Treatment with PVP showed no appreciable improvement in shoot survival as compared with the control group. These results suggest regeneration efficiency of Grosso can be greatly improved by incorporation of ascorbic acid in the medium.

Contrary to previous reports, the polyamines Spd and Spm were found to have an inhibitory effect on rooting efficiency, while Put had no significant effect on rooting. Although there was little or no effect at low concentrations, the inhibitory effect became significant with increasing polyamine concentration. Additionally although most explants looked healthy with robust shoots at low concentration levels, increased concentrations of Spd and Spm lead to marked browning and large amounts of callus growth, particularly on WPM. It has been previously reported that low levels of Put, Spd and Spm in addition to phenolic build-up in culture can lead to tissue browning (Tang and Newton 2004). Our results demonstrate that polyamines at higher concentrations may lead to tissue browning, necrosis and growth inhibition. Interestingly, although there was no significant interaction between
polyamine treatment and media type in terms of rooting efficiency, there does seem
to be a difference in overall shoot health and callus production between media types
as shoots treated with polyamines on MS media were generally healthier in
appearance than those grown on WPM media. Further studies are required to
determine the mechanism of this interaction.

In summary we have developed an efficient method for the regeneration of _L. x
intermedia_ cv Grosso from leaf tissue. The plant growth regulator TDZ efficiently
induces callus formation and shoot initiation. Although rooting, induced by IAA, has
highest efficiency under red light when full-strength media is used, shoots grown on
half strength MS and WPM media efficiently produce roots. Further, white light
promotes healthier (non-etiolated, more robust) shoots with less callus production,
and is thus preferred. The inclusion of AA minimizes browning through the culture
process, leading to improved shoot quality and survival. In contrast, application of
the polyamines Spd and Spm at concentrations above 10 μM promotes browning
and inhibits root formation in Grosso cultures. Further studies are required to
determine the mechanism of this inhibition.

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Tables

Table 1. Average vigor of lavandin cultures after exposure to various treatments, where 1 indicates a dead explant and 4 a healthy mature explant with no browning. Tukey’s HSD (α = 0.05, n = 12) was used to determine significant differences with treatments being significantly different assigned different letters, and those that are not significantly different assigned the same letter.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Vigor</th>
<th>Standard Deviation</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.42b</td>
<td>0.51</td>
<td>(0.09, 0.74)</td>
</tr>
<tr>
<td>0.5 g/L AC</td>
<td>2.58a</td>
<td>1.38</td>
<td>(1.71, 3.46)</td>
</tr>
<tr>
<td>1 g/L AC</td>
<td>2.50a</td>
<td>1.73</td>
<td>(1.40, 3.60)</td>
</tr>
<tr>
<td>0.21 mg/L AIP</td>
<td>2.67a</td>
<td>1.49</td>
<td>(1.72, 3.62)</td>
</tr>
<tr>
<td>10 mg/L AA</td>
<td>3.67a</td>
<td>0.88</td>
<td>(3.10, 4.23)</td>
</tr>
<tr>
<td>250 mg/L PVP</td>
<td>0.50b</td>
<td>0.52</td>
<td>(0.17, 0.83)</td>
</tr>
</tbody>
</table>

Legends to Figures

Figure 1. Regeneration of lavandin from leaf cuttings where: (a) callus induction, (b) shoot initiation, (c) shoot multiplication.

Figure 2. Examples of shoots classified as (a) healthy, mature shoot, health = 4 (b) mature shoot with browning, health = 2 (c) healthy, immature shoot, health =3 (d) immature shoot with browning, health = 1

Figure 3. Contingency analysis of explant vigor after exposure to various treatments, n=12 for all treatments, colour blocks represent proportional distribution of explants by health.

Figure 4. Boxplot for rooting efficiency observed after four weeks on light, media type and media strength treatments where solid bars represent the mean, boxes encompass the first and third quartiles and whiskers extend to range of data with experiments performed in triplicate and n = 4 for all treatments. Letters are assigned based using Tukey’s HSD (α = 0.05) with treatments are significantly different assigned different letters.

Figure 5. Explants after four weeks on light, media type and media strength treatments where treatments are as follows: (a) red light, half MS (b) red light, full
MS (c) white light, half MS (d) white light, full MS (e) red light, half WPM (f) red light, full WPM (g) white light, half WPM (h) white light, full WPM

Figure 6. Boxplot of rooting efficiency observed after four weeks on 10, 100 and 1000 μM polyamine treatments (Spd, Spm or Put on either MS or WPM) where solid bars represent the mean, boxes encompass the first and third quartiles and whiskers extend to range of data with experiments performed in triplicate and n = 6 for all treatments. Letters are assigned based using Tukey’s HSD (α = 0.05), with treatments that are significantly different assigned different letters. From top to bottom treatment plots are: Spd, Put, Spm.

Figure 7. Explants after four weeks on polyamine treatments (Spd, Spm or Put on either MS or WPM) where treatments are from left to right as follows: a-c 10, 100 and 100 μM Spm on MS; d-f 10, 100 and 1000 μM Spm on WPM; g-i 10, 100 and 1000 μM Spd on MS; j-l 10, 100 and 1000 μM Spd on WPM; m-o 10, 100 and 1000 μM Put on MS; p-r 10, 100 and 1000 μM Put on WPM.
Figure 5
Created in: EazyDraw3
Figure 6
Created in: R