Micropropagation of *Vitex agnus-castus*©

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INTRODUCTION

Vitex agnus-castus or the chaste tree is a large shrub that is important as a medicinal and an ornamental plant. It is well known for its medicinal properties in hormone regulations (Snow, 1996); research is actively being conducted to prove the medicinal properties of the shrub (Van Die et al., 2013). In addition to being an important herbal medicine, the shrub also possesses ornamental properties with promising marketable value (Dirr, 2015). The shrub has fragrant palmately compound leaves and produces spikes of lavender flowers in the late summer that attract pollinators (Gilman and Watson, 1994). It can grow in many types of soil with good drainage and the shrub does not attract deer. Lastly, *Vitex* is not known to be affected by any major pest or diseases.

Vitex has a few weaknesses (Dirr, 2015); the shrub has an aggressive growth habit and can grow up to 20 ft in length and width. This quality likely deters home owners and landscape architects to use *Vitex* in a landscape. Another weakness of *Vitex agnus-castus* is that it has limited flower colors. Currently the flower colors that are available commercially are limited to two: purple-blue and pink. Lastly the shrub is not cold tolerant or hardy in zones colder than zone 7 (USDA Plant Database). This limits the landscape or area that the plant can be used.

Considering the economic potential of *Vitex*, a breeding program will be able to improve *Vitex* as an ornamental plant. An important step to this goal is to outline the micropropagation protocols for this plant.

MATERIALS AND METHODS

Surface sterilization

Cuttings were taken from plants, approximately 3-5 months old, growing in the greenhouse. Cuttings were wrapped in wet paper towel and transported into the laboratory in a closed container. The apical meristem and the leaves were removed and the cuttings were cut to 7-10 cm. The cuttings were then individually washed under warm running water for 30-60 s before they were stirred in soapy solution for 30 s. The cuttings were washed under running water for another 30-60 s to remove the soapy solution. After washing, the cuttings were placed in Magenta™ boxes containing 200 ml of sterile water and placed under the laminar air-flow hood before they were soaked in 10% bleach (8.25% NaOCl) solution for 15 min. with occasional stirring every 5 min. After 15 min., the cuttings were washed three times with sterile water to remove any bleach solution. Then individual nodes with internodes removed were cultured into test tubes containing 10 mL half strength Murashige and Skoog (MS; Murashige and Skoog, 1962) nutrient medium, 30 g L^{-1} sucrose, and 6 g L^{-1} agar micropropagation grade (Phytotech Laboratories®) with pH calibrated to 5.70-5.75. The explants were then placed under florescent lights and allowed to grow for 4 weeks. Contamination and survivability of explants were recorded after 4 weeks (Table 1).

Shoot multiplication

For shoot multiplication media, full strength MS medium was prepared with 30 g L^{-1} sucrose, 6 g L^{-1} agar and the pH calibrated to 5.70-5.75 before the medium was autoclaved. The medium prepared was poured into Magenta™ boxes with 40 mL box^{-1} and 5 explants were cultured into each box.

The explants used for the experiment were maintained in full strength MS media. The

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explants were cut into approximately 2.5 cm in length with the apical meristem removed to remove apical dominance. Twenty explants were used for each treatment with a total of 10 treatments including the control. The treatments were control (no growth regulators), 0.1 mg L^{-1} BA, 0.5 mg L^{-1} BA, 1.0 mg L^{-1} BA, 5.0 mg L^{-1} BA, 0.1 mg L^{-1} 2iP, 0.5 mg L^{-1} 2iP, 1.0 mg L^{-1} 2iP, and 5.0 mg L^{-1} 2iP. The explants were allowed to grow for 4 weeks before the number of shoots and the length of shoots produced were recorded.

Rooting

For rooting media, full strength MS medium was prepared with 30 g L^{-1} sucrose, 6 g L^{-1} agar and the pH was calibrated to 5.70-5.75 before autoclaved. Twenty explants were used for each concentration tested and the explants were maintained in full strength MS medium supplemented with 1 mg L^{-1} BA. The explants were cut to 2.5 cm in length, each with the apical meristem included on the explant. The explants were cultured in medium supplemented with either IBA or NAA at concentration of 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1} and 5.0 mg L^{-1}. Five explants were cultured into one Magenta™ box and they were allowed to grow for 4 weeks before the number of explant rooted and the length of primary roots produced were measured.

Acclimatization

Rooted explants that were maintained in MS medium supplemented with 1 mg L^{-1} IBA were used for the experiment. The explants were washed under running water to remove tissue culture medium before they were potted into Magenta™ boxes containing sterile 50:50 mix of perlite and peat moss. Four explants were placed inside each box and thin transparent plastic bags were used to loosely cover the top of the magenta boxes. The explants were then placed under fluorescent light for 4 weeks. The explants were watered with 15-ml sterile water on the first day and every 2 days after or as needed. After 1 week, a vertical slit with 0.5 cm in length was made on the plastic cover and the length of the opening was doubled every 4 days. On the 4th week, after 2 days with the opening of the slit to be 8 cm, the explants were taken to the greenhouse and potted into 4-inch pots and were placed under shade cloth. The survival of the explants was recorded at the end of the 5th week.

RESULTS

Surface sterilization

Table 1 summarizes the result of surface sterilization method. Cuttings taken from the greenhouse were sterilized before they were cultured individually in test tubes. Fifty nodes were obtained from the sterilized cuttings and after 4 weeks in culture, three test tubes were observed to be contaminated. The success of the procedure was calculated to be 94%.

Table 1. Result of surface sterilization method on *Vitex* plants; cuttings were successfully introduced into sterile environment with 94% success rate.

Number of explants/nodes	Number of contaminated explants	Percent success
50	3	94

Shoot multiplication

1. BA media.

Figures 1 and 2 show the results of 20 explants grown on different BA concentrations for four weeks. The explants in the control medium produced an average of 2.4 shoots at 1 cm in length while the medium supplemented with 0.1 mg L^{-1} BA produced an average of 3.2 shoots at a length of 1.1 cm. Medium supplemented with 0.5 mg L^{-1} BA produced 5.6 shoots at 1.1 cm in length while medium with 1.0 mg L^{-1} BA produced the greatest number of shoots

with 7.5 shoots at 1 cm in length. Lastly, the 5.0 mg L^{-1} BA medium produced 6.2 shoots on average at 0.6 cm in length.

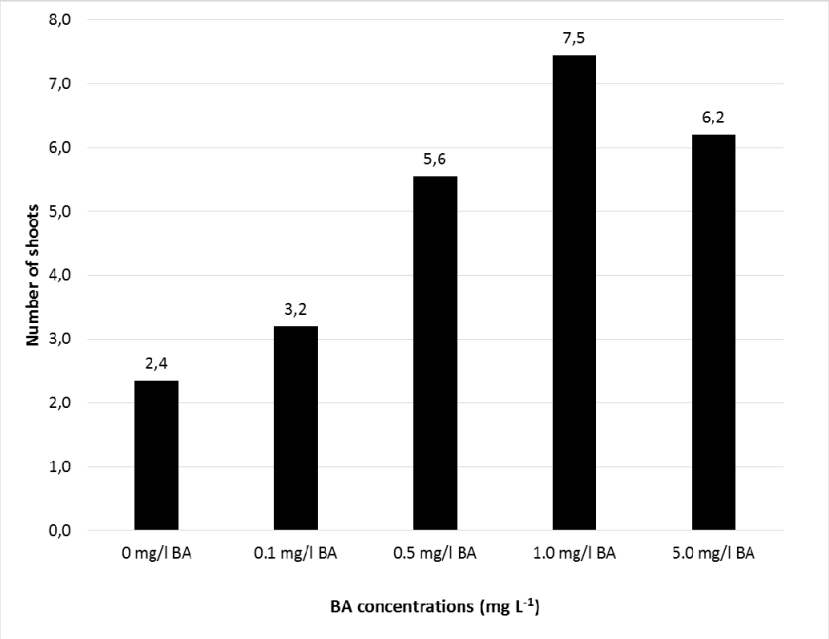

Figure 1. Average number of shoots produced when *Vitex* explants were grown in different BA media after 4 weeks.

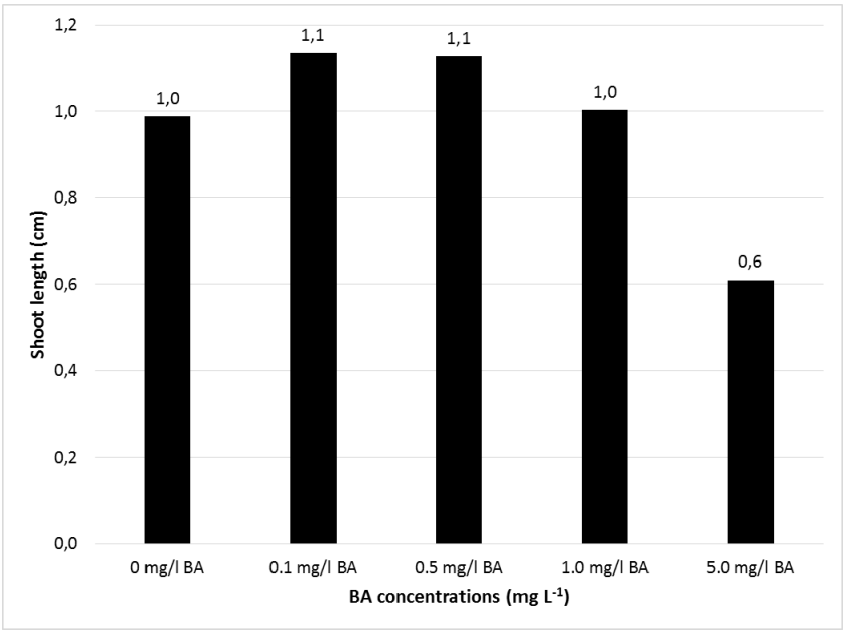

Figure 2. Average shoot length of *Vitex* cuttings when explants grown in different BA media after 4 weeks.

2. 2iP media.

Figures 3 and 4 show the result of 20 explants grown in different 2iP concentrations for 4 weeks. The control medium produced an average of 2.7 shoots at 2.7 cm in length, 0.1 mg L^{-1} 2iP medium produced 3 shoots with length of 1.4 cm, 0.5 mg L^{-1} 2iP medium

produced 3.8 shoots with length of 1.3 cm, 1.0 mg L^{-1} 2iP medium produced 3.8 shoots with length of 0.8 cm and 5.0 mg L^{-1} 2iP medium produced 5.0 shoots with length of 0.9 cm.

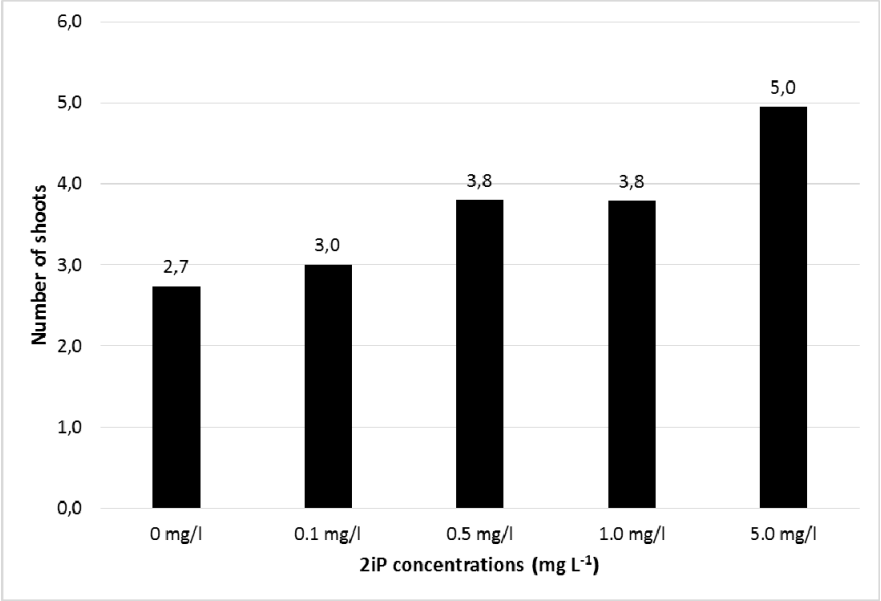

Figure 3. Average number of shoots produced on *Vitex* explants when grown in different 2iP media after 4 weeks.

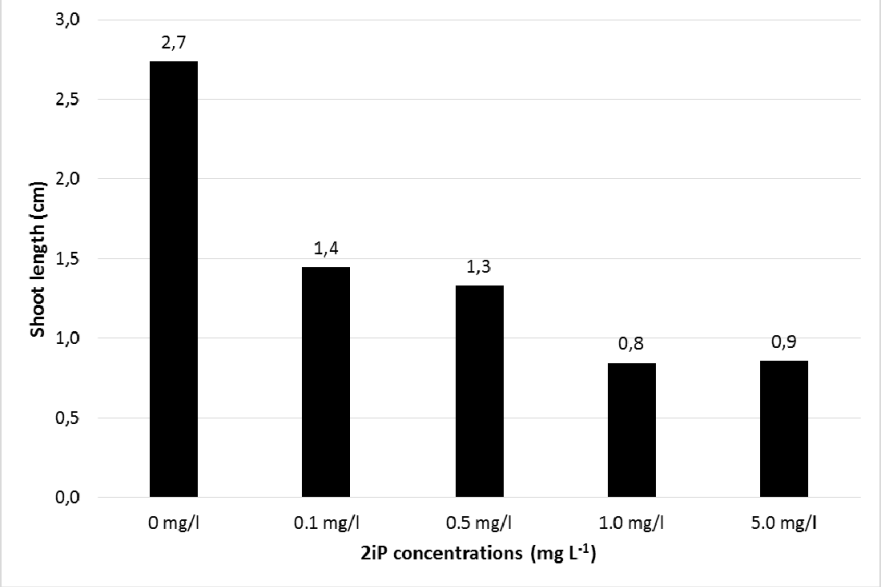

Figure 4. Average shoot length on *Vitex* explants when grown in different 2iP media after 4 weeks.

Rooting

1. IBA media.

Twenty explants of *Vitex* were cultured in media supplemented with different concentrations of IBA for four weeks. The control medium had 30% of explants rooted with root length of 0.5 cm, while 0.1 mg L^{-1} IBA had 35% explants rooted with length of 0.8 cm (Figure 5). The medium supplemented with 0.5 mg L^{-1} IBA had the greatest number of

rooted explants with 65% explants rooted with the root length of 1.8 cm; 1.0 mg L^{-1} IBA and 5.0 mg L^{-1} IBA media both had 55% rooted explants with root length of 2.3 cm and 2.1 cm, respectively (Figure 6).

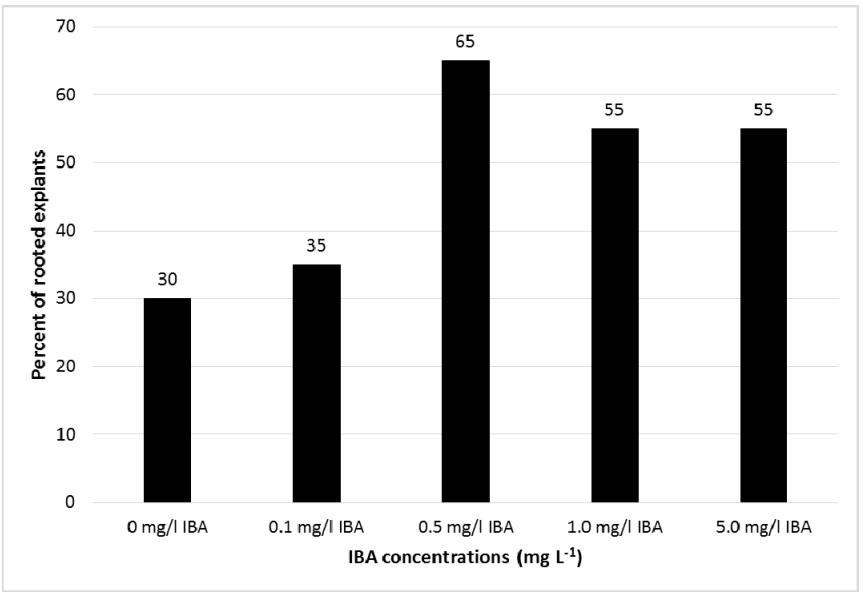

Figure 5. Percentage of *Vitex* explants that rooted after growing in different IBA media for 4 weeks.

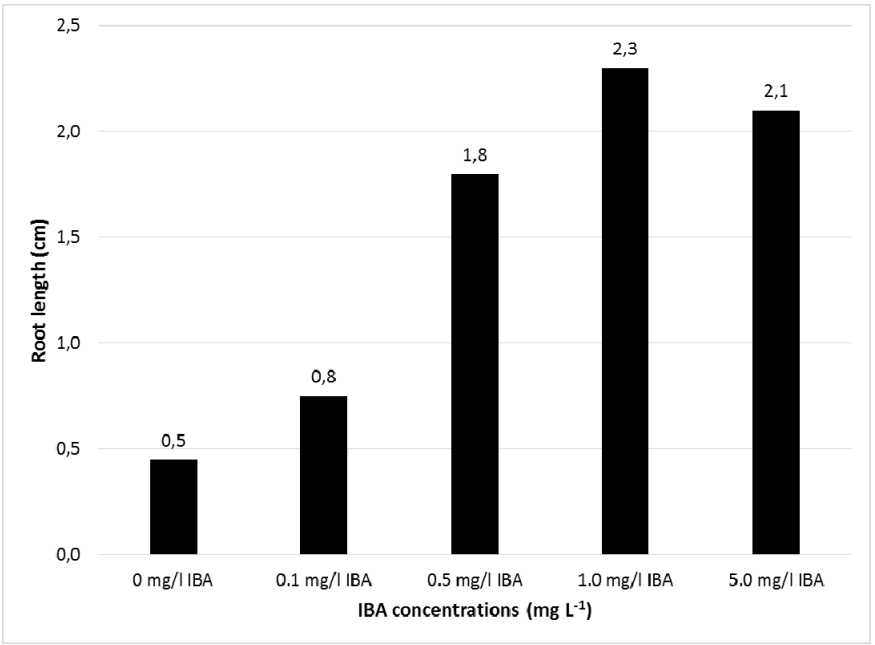

Figure 6. Average root length produced by *Vitex* explants that were grown in different IBA media after 4 weeks.

2. NAA media.

Twenty *Vitex* explants were cultured in media supplemented with NAA at different concentrations for four weeks. The control medium and the 0.1 mg L^{-1} NAA medium both had 35% explants rooted with 1.4 cm and 0.9 cm root length, respectively (Figure 7). The 0.5 mg L^{-1} NAA medium had 40% explants rooted with length of 1.3 cm while 1.0 mg L^{-1} NAA

medium had only 30% explants rooted at 0.7 cm in length. The greatest number of explants rooted was observed in the medium supplemented with 5.0 mg L^{-1} NAA with 65% explants rooted with 2.4 cm root length (Figure 8).

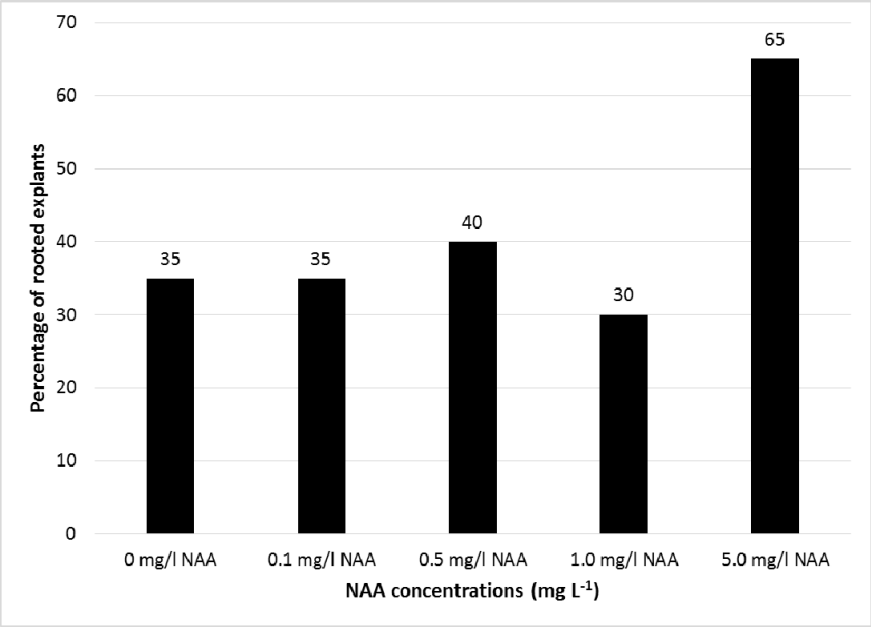

Figure 7. Percentage of rooted *Vitex* explants after 4 weeks of growing in different NAA media for 4 weeks.

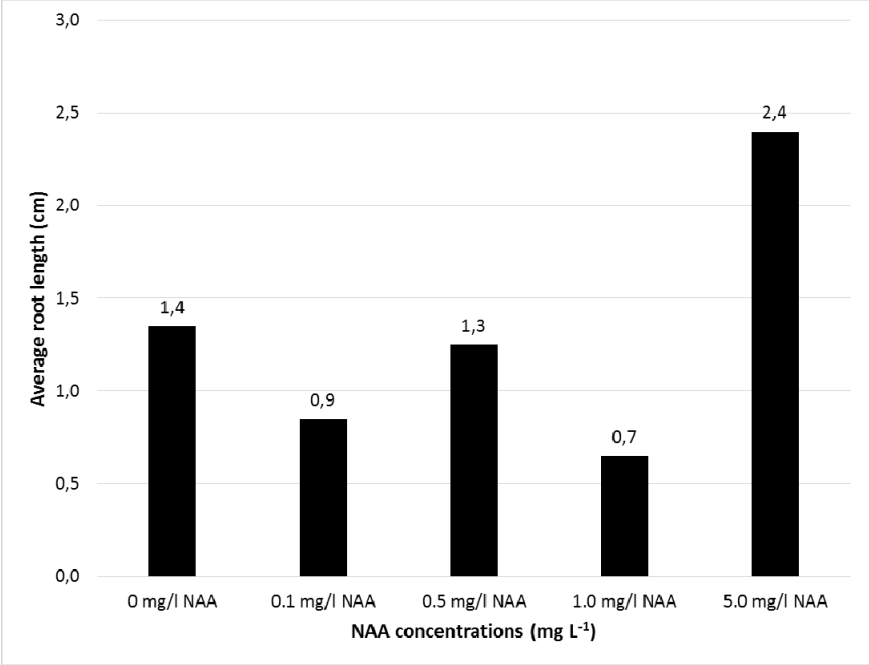

Figure 8. Average root length produced by *Vitex* explants grown in different NAA media after 4 weeks.

Acclimatization

Table 2 summarized the result of the acclimatization protocol. Twenty rooted explants were used for the experiment and 17 explants were successfully transferred into the

greenhouse with a survival percentage of 85%.

Table 2. Twenty rooted explants were used for acclimatization experiment with 85% success rate.

Number of explants	Media	Number of survival explants after 5 weeks	Survival percentage
20	50:50 perlite:peat moss	17	85

DISCUSSION

Surface sterilization

There were two objectives for the surface sterilization experiment. The first objective was to eliminate any contaminants on the cuttings and the second objective was to ensure the survival of the explants that undergo the sterilization treatment. The contaminants on the cuttings' surface were eliminated through physical and chemical methods. The contaminants were physically removed from the surface of the cuttings by washing the cuttings under running water combined with stirring the cuttings in the soapy solution. After physically removing contaminants from the surface, 10% bleach solution soaked was used to further decontaminate the surface of the cuttings.

The surface sterilization method successfully sterilized 47 nodes out of 50 nodes from the cuttings taken from the greenhouse. The success rate was reported to be 94% and the nodes that were successfully sterilized started growing new shoots after 3 weeks in tissue culture.

The container used in the experiment contributed to the success rate of sterilizing the cuttings. Prior experiments of surface sterilization used Magenta™ boxes with 5 nodes cultured into one box and the success rate was much lower as compared to using test tubes (data not included). The use of test tubes allowed physical isolation of explants after the sterilization method. When magenta boxes were used, contamination from one explant will result in the loss of five explants that were in the same container. Thus, the use of test tubes prevents any recalcitrant spores or contaminants that were not removed during the sterilization procedure to spread to other explants.

Shoot multiplication

Two phytohormones were compared to determine the optimum cytokinin for shoot multiplication of *Vitex*. The explants grown in the control media contained no phytohormone supplement and produced on average of 2.4 shoots. The media supplemented with 0.1 mg L^{-1} BA produced 3.2 shoots, 0.5 mg L^{-1} of BA produced 5.6 shoots, 1.0 mg L^{-1} of BA produced 7.5 shoots and the highest concentration of BA tested of 5.0 mg L^{-1} produced 6.2 shoots after 4 weeks of growth. The average length of shoots produced for each of the concentrations tested were 1.0 cm for the control, 1.1 cm for 0.1 mg L^{-1} BA, 1.1 cm for 0.5 mg L^{-1} BA, 1.0 cm for 1.0 mg L^{-1} BA and 0.6 cm for 5.0 mg L^{-1} BA.

For explants grown in media supplemented with 2iP, 0.1 mg L^{-1} 2iP produced 3.0 shoots, 0.5 mg L^{-1} 2iP produced 3.8 shoots, 1.0 mg L^{-1} 2iP produced 3.8 shoots and 5.0 mg L^{-1} 2iP produced 5.0 shoots on average. As for the average length of shoots produced on 2iP supplemented media, shoot length was 1.4 cm for 0.1 mg L^{-1} 2iP, 1.3 cm for 0.5 mg L^{-1} 2iP, 0.9 cm for 1.0 mg L^{-1} of 2iP and 0.9 cm for 5.0 mg L^{-1} of 2iP.

The supplemented phytohormones used in the experiment promoted shoot production. The number of shoots in the supplemented media produced more shoots as compared to explants grown in the control media. The length of shoots that are produced decreased as the amount of phytohormone was increased. This is expected as the explants were forced to divert energy expenditure towards shoot production instead of shoot elongation.

The greatest number of shoots produced from media supplemented with BA was 7.5 shoots at the concentration of 1.0 mg L^{-1} and the shoot length is 1 cm on average. The

greatest number of shoots produced from media supplemented with 2iP was 5.0 shoots at concentration of 5.0 mg L^{-1} with the average shoot length of 0.9 cm. This showed that BA was better at promoting shoot production compared to 2iP and the optimum BA concentration to be used to for shoot production was 1.0 mg L^{-1}.

Rooting

In the control media with no auxin supplement, 30% of explants formed roots after 4 weeks of growth. With media supplemented with IBA, 0.1 mg L^{-1} IBA had 35% rooted explants, 0.5 mg L^{-1} IBA had 65% rooted explants and both 1.0 mg L^{-1} IBA and 5.0 mg L^{-1} IBA had 55% rooted explants each. The average length of roots produced in the control media was 0.5 cm, 0.8 cm for 0.1 mg L^{-1} IBA, 1.8 cm for 0.5 mg L^{-1} IBA, 2.3cm for 1.0 mg L^{-1} IBA and 2.1 cm for 5.0 mg L^{-1} IBA.

For media supplemented with NAA, 0.1 mg L^{-1} NAA had 35% rooted explants, 0.5 mg L^{-1} NAA had 40% rooted explants, 1.0 mg L^{-1} NAA had 30% rooted explants and 5.0 mg L^{-1} NAA had 65% rooted explants. The average length of roots produced were 0.9 cm for 0.1 mg L^{-1} NAA, 1.2 cm for 0.5 mg L^{-1} NAA, 0.7 cm for 1.0 mg L^{-1} NAA and 2.4 cm for 5.0 mg L^{-1} NAA.

Based on the data obtained, the auxins used in the experiment promoted rooting in the explants since more explants rooted in media supplemented with auxins as compared to the explants grown in the control media. The concentration of IBA with the highest percentage of rooted explants was 0.5 mg L^{-1} with 65% explants rooted after 4 weeks of growth with an average root length of 1.8 cm. for NAA. The concentration that had the highest percentage of rooted explants was 5.0 mg L^{-1} NAA with an average root length of 2.4 cm. it can be concluded that the auxin IBA is more effective at inducing root production in *Vitex agnus-castus* as compared to NAA since IBA was able to achieve 65% rooting at concentration of 0.5 mg L^{-1} while it requires 5.0 mg L^{-1} NAA to achieve the same percentage of rooted explant.

Acclimatization

Acclimatization of explants was achieved by controlling the humidity of the growing environment. Rooted explants were taken off tissue culture media and the roots were washed thoroughly to avoid any fungal growth from any sucrose residue. During the first week of acclimatization, the humidity of the growing environment was not altered and the explants were allowed to adjust to the new growing condition without sucrose supplement. This allowed the explants to start or increase its photosynthetic machinery. In the second week of acclimatization, the humidity of the growing environment was decreased starting with a vertical slit of 0.5 cm and the length of the slit was doubled every 4 days to gradually decrease the humidity of the growing environment. Some damaged leaves were observed during the second week but the damage was minimal.

From the 20 explants tested for the acclimatization experiment, 17 explants were successfully introduced into the greenhouse. In the greenhouse, the plants were placed under shade cloth to limit light exposure before they are exposed to full sunlight.

SUMMARY

The experiments designed in the research achieved their goals. The surface sterilization method successfully introduced greenhouse cuttings of *V. agnus-castus* into sterile environment with 94% success rate. For shoot multiplication, the cytokinin BA proved to be more effective compared to 2iP and the optimum concentration to use was 1.0 mg L^{-1} of BAP. To induce rooting, the auxin IBA proved to be more effective compared to NAA and the optimum concentration to use was 0.5 mg L^{-1} with 65% explants rooted after 4 weeks. Lastly the acclimatization protocol successfully introduced 85% of rooted tissue culture explants into the greenhouse.

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