In vitro propagation and re-introduction of Golden Paintbrush (*Castilleja levisecta*), a critically imperiled plant species

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| **Complete List of Authors:** | Salama, Ahmed; University of Guelph, Plant Agriculture  
Shukla, Mukund; University Of Guelph, Plant Agriculture  
Popova, Elena; Global Crop Diversity Trust  
Fisk, Nathan; Park Canada  
Jones, M.; University of Guelph, Plant Agriculture  
Saxena, Praveen; University of Guelph, Plant Agriculture |
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In vitro propagation and re-introduction of Golden Paintbrush (*Castilleja levisecta*), a critically imperilled plant species

Ahmed Salama¹, Mukund R. Shukla¹, Elena Popova², Nathan S. Fisk³, Maxwell P. Jones¹, Praveen K. Saxena¹

¹Gosling Research Institute for Plant Preservation, Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada.
²Global Crop Diversity Trust, Bonn 53113, Germany
³Parks Canada / Government of Canada, 603 Fort Rodd Hill Rd., Victoria BC V9C 2W8

*Corresponding author: psaxena@uoguelph.ca
ABSTRACT

Golden paintbrush (*Castilleja levisecta* Greenm.) is a hemi-parasitic herbaceous perennial native to the Pacific Northwest of North America and is considered critically imperilled with only 11 populations remaining in the wild. The main objective of this study was to develop ex situ and in situ conservation through micropropagation and field plantings. In vitro cultures were initiated using nodal explants from two plants raised from seeds collected from a natural population. Shoots were then multiplied on MS basal medium with 2.0 µM BA, 3.0 µM Kinetin, 2.2 g/L phytagel and 3% sucrose. Explant position on source plants, culture vessel design and application of different plant growth regulator levels for 6-Benzylaminopurine (BA), Kinetin (Kn) and Thiadiazuron (TDZ) were tested in order to optimize micropropagation protocols. Clones from the plants showed differences in plant height and number of nodes in response to various BA and TDZ concentrations. In vitro shoots were successfully rooted under ex-vitro conditions using commercial rooting powder (0.8% Indole-3-Butyric Acid) with an average of ~17 roots per shoot and acclimatized in the greenhouse with 100% survival rate. Two month old plants were transferred to a Parks Canada restoration site at Fort Rodd Hill, BC, Canada with 7.5% survival. The use of micropropagation in combination with re-introduction efforts offers an excellent opportunity for conserving endangered plant biodiversity in vitro and facilitating in situ conservation efforts by providing plants for re-introduction.

**Keywords:** Golden paintbrush, *Castilleja levisecta*, micropropagation, endangered, restoration
INTRODUCTION

*Castilleja levisecta* Greenm. (Orobanchaceae, formerly in Scrophulariaceae is a short-lived perennial (5–6 years), and commonly referred to as ‘Golden paintbrush’ as the golden yellow bracts effectively hide its greenish flowers. Recent surveys suggest that *C. levisecta* has lost most of its historic range and there are only 11 known populations remaining (Gamon 1995). The Nature Conservancy has ranked golden paintbrush as G1 - globally critically imperiled and it is listed as Threatened under the U.S. Endangered Species Act. In 2000, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) ranked golden paintbrush as endangered based on a report prepared in 1995 (COSEWIC 2007). Given the recovery plan for *C. levisecta* emphasizes reintroduction as an important activity for its conservation (Caplow, 2004), maintenance of current population sizes is critically important to prevent the loss of genetic diversity within the species.

Golden paintbrush exclusively reproduces by seed in nature. However, poor seed germination and early seedling mortality makes it difficult for this species to propagate by seeds (Pearson and Dunwiddie 2006). *C. levisecta* has limited capacity for natural dispersal and colonization of new sites, necessitating ex situ conservation to meet recovery goals (USFWS 2000). Low seed germination and occurrence within a restricted habitat has also prevented the use of conventional ex situ conservation efforts such as seed banking for this species. Ex situ in vitro conservation has been used for many plant species and is especially important for critically endangered species with small populations (Goncalves et al., 2010; Mallon et al., 2010). To date clonal propagation through asexual reproduction has been unsuccessful (Dunwiddie et al. 2001). Furthermore, micropropagation of *C. levisecta* has not been reported, thus the objective of the current study was to develop an efficient protocol for in vitro mass propagation of *C. levisecta*.
and to redistribute micropropagated individuals back into their natural habitat. This represents, to the best of our knowledge, the first application of micropropagation technologies for field redistribution to restore *C. levisecta* populations, an endangered species in Canada.

**MATERIALS AND METHODS**

**Seed Germination and viability**

Seeds from plants grown in the Fort Rodd Hill Conservation Nursery, Victoria, British Columbia were collected in the fall of 2016 and stored at 4°C for six months (Fig. 1A). Nearly 300 seeds from 6 seedpods were surface sterilized with 10% bleach for 15 minutes, and rinsed with autoclaved deionized water four times for 3 minutes each. Seeds were plated on semi-solid medium consisted of MS (Murashige and Skoog 1962) basal salts with vitamins (Phytotechnology, Shawnee Mission, Kansas, USA), 3% sucrose, and 2.2 gL⁻¹ phytagel (Sigma-Aldrich, Canada). The pH of the medium was adjusted to 5.7 before autoclaving for 20 minutes at 121°C and 118 kPa. A total of 12 Petri dishes with an average 25 seeds on each plate were kept in the dark for 10 days and moved to the growth chamber at 25 ± 2 °C under a 16 h photoperiod provided by cool white fluorescent lamps (Osram Sylvania Ltd., Mississauga, Ontario) at light intensity of 35-40 µmol·m⁻²·s⁻¹. Nearly 150 seeds in three groups were used for seed viability test with 1.0 % tetrazolium chloride test. Germination percentage was recorded after 4 and 10 weeks of culture.

**Plant material**

Seeds collected from wild plants were grown for approximately two months at Fort Rodd Hill, Victoria, British Columbia. They were then shipped to the University of Guelph (Guelph, ON) where they were maintained on a mist bed in the greenhouse. To initiate in vitro cultures, 5 nodal explants were taken from the two plants and surface sterilized using a 10% commercial
bleach solution (Clorox®; 5.4% sodium hypochlorite) with two or three drops of Tween 20 for 7 min, followed by three rinses in sterile distilled water for 3 minutes each time. The explants were then transferred to Magenta GA7 culture vessels containing shoot development medium (SDM) composed of MS basal salts, Gamborg’s B5 (Gamborg et al. 1968) vitamins, 2.0 µM 6-benzylaminopurine (BA), 3.0 µM Kinetin (Kn) (all obtained from Phytotechnology, Shawnee Mission, Kansas, USA), 3% sucrose, and 2.2 g L⁻¹ phytage (Sigma-Aldrich, Canada). In vitro plants were multiplied using single node sections (Fig. 1C) sub-cultured every six weeks. All cultures were maintained in the culture room with the same conditions specified for seed germination. Clones of the two seedlings were maintained separately and labeled as G1 and G2. These nodal segments were subsequently used for multiplication of shoots and experimental materials.

In vitro propagation

To optimize shoot growth and multiplication rate, single nodes of similar size from six-week-old plants were sub-cultured onto MS basal medium supplemented with different concentrations of BA and TDZ (0, 0.1, 1.0, 10 µM) and compared with shoot development medium (SDM). Other experiments were conducted using nodal explants from the G2 clone in order to observe the effect of combining BA and Kn together at four concentrations (including SDM) on shoot multiplication (Table 1). Each treatment was replicated three times and consisted of 12 test-tubes with 10 ml of medium, and a single nodal explant in each. Shoot length and the number of nodes on each explant were recorded after four weeks of culture. After comparing the media for shoot growth and development, six week old plantlets were used for further evaluation of explant position using nodal explants from positions 1 to 10. Similarly, single nodes sub-culture onto the SDM in Petri dishes (Polystyrene disposable sterile, 100*25mm, VWR
CATALYST, 30ml media), Test tubes (15 ml media), and Magenta GA7 vessels (50 ml media) was performed to evaluate the effect of culture vessel on shoot development and multiplication rate. All nodes from a single shoot were cultured in the same Petri dish or Magenta GA7 vessel and labelled based on their position on the plant. In the case of tubes, each node was cultured separately and the tubes were labelled based on the node position. Three sets of vessels were considered as a replication and a total of three replications were used in this experiment. Observations related to growth for each node were recorded after two weeks of culture.

**In vitro rooting**

Experiments were conducted in Magenta GA7 vessels in order to test the extent to which growth regulators effect rooting under in vitro conditions. Each treatment consisted of five Magenta GA7 vessels with 50 ml of medium (four explants/vessel), and was replicated three times. Six week old in vitro shoots of golden paintbrush (6-7cm long) with 6-8 nodes were then sub-cultured on MS basal medium supplemented with various concentrations (1.0, 5.0 and 10.0µM) of Indole-3-Butyric Acid (IBA) or Naphthalene Acetic Acid (NAA). The shoots were maintained in the culture room at 25 ± 2 °C under a 16 h photoperiod with a light intensity of approximately 40 µmol·m⁻²·s⁻¹. The observations were recorded after 3 weeks for root development.

**Ex vitro rooting and acclimatization in a greenhouse**

In order to assess the potential of ex vitro rooting, two-month-old shoots were transferred from in vitro conditions directly into potting mix and maintained on a mist bed (24°C; 14 h light and 20°C for 10 h darkness, over 85% humidity). In vitro developed shoots were gently rinsed with tap water to remove culture medium before immersing the bottom part of stems (~ 1 cm long) in various commercial rooting powders (Stim-Root # 1, 2, and 3, Plant Products Co. Ltd.,
ON, Canada) containing three concentrations of IBA (0.1, 0.4, 0.8%, respectively). Shoots were immediately planted in plastic containers filled with pre-moistened growth medium (Sunshine mix - 4; Sun Gro Horticulture Canada Ltd., Vancouver, Canada) and kept in the mist bed for four weeks. Shoots planted without auxin treatment were included as a control. Experiments were conducted with three replications and 12 shoots used per replication. Root length and number of roots were recorded after four weeks of planting.

Reintroduction in natural habitat

In March 2016, 268 plants developed through micropropagation were shipped through FedEx® overnight service to the Conservation Nursery in Fort Rodd Hill, Canada (clone line – G1). Upon arrival plants were kept in low light/wind conditions for 7 days to acclimatize to local conditions. They were then co-planted with *Eriophyllum lanatum* as a host plant in raised beds. Survival of plants was assessed over 3-5 months by visual cues including stem elongation, appearance of new leaves, and flowering.

Statistical analysis

All experiments were arranged in a completely randomized design and repeated three times. Data were analyzed by ANOVA, followed by the Duncan’s Multiple Range Test at $p = 0.05$ using SAS University edition 3.4 (SAS Institute Inc. Cary, North Carolina, USA). Figures present mean values with standard error. Values followed by different letters are significantly different at $p< 0.05$.

RESULTS:

Seed Germination and Viability

Very low germination rate (~5%) was observed on semi-solid medium (Fig. 1B).
However, seeds stored at 4°C showed nearly 97% seed viability based on a 1.0% tetrazolium chloride test.

**Culture initiation and in vitro plant propagation**

Golden paintbrush plants were successfully introduced to in vitro conditions using nodal explants from seedlings received from British Columbia (Fig. 1C). The majority of axenic nodal explants initiated new growth within 1-2 weeks of culture, but approximately 40% were visibly contaminated and discarded. After four weeks in culture, segments from both clones (G1 and G2) developed tall, healthy shoots with 3-4 internodes (Fig. 1D).

In order to evaluate the possibility of further improvement in shoot multiplication and growth, various levels of BA and TDZ were used in additional experiments. Both accessions of golden paintbrush responded for shoot growth to varying levels of plant growth regulators (BA and TDZ), (Fig 2). Shoots of G1 clone in the medium with BA (1.0 µM) developed the greatest number of nodes (8.25) compared to other treatments of BA, TDZ, or SDM medium (Fig 2), with the exception of 0.1 µM BA and the control. However, in the case of clone G2, the SDM treatment produced shoots that were significantly taller with greater number of nodes (11.05) followed by the treatment with 0.1µM TDZ (Fig 2).

In the experiment evaluating BA and Kn in combination, G2 clones showed the greatest average shoot height (3.19 cm) and the average number of nodes (6.0) on SDM (2.0 µM BA & 3.0 µM Kn). However, plant height in this treatment was only significantly different from the 3.0 µM BA & 2.0 µM Kn treatment (Table 1). In terms of the number of nodes, there were no significant differences observed between SDM and 4.0 µM BA & 1.0 µM Kn µM treatments (Table 1).

In this study, three culture vessels and 10 explants based on node position on mother
plants were studied for their effects on shoot growth and development. Overall, culture vessel design and nodal position showed a significant interaction effect on shoot development (Fig S1a). For instance, more explants developed shoots in test tubes (89%) and Petri dishes (88%) than in magenta boxes (Fig S1b). The nodes from the third position from the top showed 100 percent shoot development while the 5th and 6th node position developed shoots at a significantly lower rate of 55% in Magenta GA7 boxes.

In vitro shoots did not respond to IBA or NAA treatments during root initiation as none of the treatments showed root induction, including the control plants (Fig 1E and F). Some plants produced pale, thin roots with what appeared to be haustoria, but were unable to survive when transferred to greenhouse conditions. However, when plants were transferred to a mist bed after being treated with different rooting powder, 100% de novo root formation was recorded in all treatments with an average of 17 roots per shoot, and there was no significant difference observed among the rooting powder treatments (Fig 1G). However, all rooting powder treatments were significantly better in respect to average numbers of roots per shoot compared to control plants (Fig 3). In vitro shoots produced significantly longer roots in the control group (4.64 cm) and those treated with 0.8% IBA (4.5 cm) as compared to other two treatments with rooting powders (Fig. 3).

All plants were successfully acclimatized ex vitro as confirmed by visible stem elongation and formation of new leaves (Fig. 1H and I). Field survival of micropropagated plants was tested with 268 plants transported to the Conservation Nursery in Fort Rodd Hill, Canada (clone line – G1). These plants were grown along with Eriophyllum lanatum as a host plant in raised beds. Of the 268 plants, 153 plants survived the transit, but many withered shortly after planting, possibly due to lack of further root development in their new environment. After nearly
five months, at least 20 plants survived this transit from lab to field and some of them had started
flowering (Fig. 1J).

**DISCUSSION:**

Ex situ in vitro conservation has been used for many plant species and is especially important for critically endangered species with small populations (Goncalves et al., 2010; Mallon et al., 2010). Golden paintbrush is an endangered species in Canada and the USA. Low seed germination and occurrence within a restricted habitat has prevented the use of conventional ex situ conservation efforts like seed banking for this species. Wentworth (1994) found poor seed germination under natural conditions, with 8% in the first year and only 2% in the second year. Moreover, the success of direct seeding resulted in 0.2 to 1 percent germination rate and very few of these seedlings survived to flower in the second season (Pearson and Dunwiddie 2006). The recovery plan for *C. levisecta* has given more emphasis on reintroduction as an important activity for conservation, but this is hampered by low germination rates (Caplow, 2004). The technology developed here represents an alternative approach to help increase plant numbers and restore populations of this endangered species.

Successful bud initiation (100%) was achieved on semi-solid medium containing MS basal salt mixture; MS based media have been shown to be suitable for various other herbaceous plant species (Sarropoulou & Maloupa, 2017; Spinoso-Castillo et al., 2017). Cytokinins can affect plant growth in a variety of ways, but are recognized as a requirement for cell division and differentiation, while also being widely used to promote shoot multiplication. However, the effects observed will vary by cytokinin type and concentration, with reported effects being species specific (Nabors, 2004; Park et al., 2008). In golden paintbrush, use of the shoot development medium (SDM) in the presence of 2.0 µM BA & 3.0 µM Kn was found to be the
best of all tested combinations for inducing shoots from nodal explants. This media was also superior compared to MS basal medium without plant growth regulators or in the presence of individual cytokinins at various levels. The SDM hormone complement (Murch et al. 2008) is routinely used to maintain and multiply a range of species in our lab.

The two accessions performed differently for numbers of nodes in response to both the cytokinins, presumably due to genetic or physiological variations within the populations. Both BA and TDZ have been used successfully for shoot multiplication in a variety of other plants including *C. tenuiflora* (Martínez-Bonfil et al., 2011), *Piper longum* L. (Rani and Dantu 2012), *Vigna subterranea* (L.) Verde (Silue et al., 2016), *Primulina tamiana* (B.L.Burtt) Mich.Möller & A.Weber (Padmanabhan et al. 2015) and *Saintpaulia ionantha* Wendl (Shukla et al. 2012).

This response could be explained by the explants endogenous hormone content whose balance would probably be in favor of cytokinins (Silue et al., 2016). Reports on in vitro culture of plants in the *Orobanchaceae* family show that plant responses vary among species. For example, experiments involving *C. applegatei* showed that the best results were obtained in medium supplemented with zeatin (Backes and Hoch, 2010), while BA was optimal for *Scrophularia yoshimurae* T. Yamaz. and *Bacopa monniera* (L.) Wettst (Sagare et al., 2001; Tiwari et al., 2001). Furthermore, Kn was found most effective in *C. tenuiflora* (Martínez-Bonfil et al., 2011).

The explant position or culture vessels have been found to impact in vitro shoot growth and proliferation (Joshi and Dhar 2003). The effect of node position on mother plants was reported in *Ponerorchis graminifolia* Rchb.f and *Capsicum annuum* L. (Mitsukuri et al., 2009; Mok & Norzulaani, 2007). Similarly, nodal position showed a significant effect on regeneration in *Commiphora wightii* (Am.) Bhandari, an endangered medicinal plant. In general, all nodes from the same mother plant produced shoots in all treatments. This observation could be
explained by the explant’s endogenous hormone content and headspace within culture vessels. Plants growing in test tubes stayed healthier for 8 weeks compared to others. This observation may be attributed to the fact that plants growing in test tubes have greater support to grow vertically by leaning against the wall of tubes while also acquiring more light and nutrition since each plant is grown individually and does not need to compete with other plants. Effect of vessels size on shoot proliferation was reported in *Vitis vinifera* L. (Monette, 1983). While the present experiments do not demonstrate that the type of vessel used is necessarily the only factor responsible for observed differences in shoot growth, they do indicate that culture vessel is important to attain better plant growth and development in vitro. Since the physical properties of culture vessels are different, the differences in shoot growth may reflect differences in gas exchange (Monette, 1983; Hazarika, 2006), humidity (Chen, 2004), transmittance distribution, and spectral irradiance (Huang and Chen, 2005). Furthermore, the gas atmosphere in plant tissue cultures is known to contain various volatiles which affect plant growth and development including ethylene (Thomas and Murashige, 1979; Hazarika, 2006).

Shoots from in vitro cultures further sub-cultured on auxin (IBA and NAA)-supplemented media failed to develop well-formed roots and hence this study explored the possibility of ex vitro rooting for whole plant recovery. In vitro rooting results were similar to those reported for *Malus* and papaya where the rooting was inhibited by IBA and NAA (De Klerk et al., 1997; Teo & Chan, 1994). Plants grown in the greenhouse developed longer, thicker and more lateral roots and produced normal plant growth. Ex vitro rooting was also found more efficient in other studies with *Rhododendron ponticum* L. and *Castilleja tenuiflora* Benth., *Centaurea ultreiae* Silva Pando, and *Pueraria tuberosa* (Willd.) DC. (Almeida et al., 2005; Martínez-Bonfil et al., 2011; Mallon et al., 2010; Rathore and Shekhawat, 2009). Ex vitro
rooting can reduce costs and increase the efficiency of the micropropagation procedure by reducing the number of steps required to induce rooting, providing a high survival rate during acclimatization (Hazarika, 2006).

After nearly five months, at least 20 micropropagated plants survived transplanting in the field, with some even flowering. Low survival of in vitro produced golden paintbrush plants in their natural habitat may be due to adverse conditions encountered during long transportation from Ontario to British Columbia. Still, a major issue affecting the survival of plants in the field may be the sensitivity of root systems which in turns renders plants unable to cope with transplant shock and stress, have poor absorption of water, and demonstrate incompatibility or lack of resistance to microbial population in the root zone or surrounding environment. Based on the above, it is likely that survival rates can be improved through adequate preconditioning of micropropagated plants with plant growth regulators and mycorrhizae. Still, the fact that a small population survived and reached flowering stage is promising as these results indicate that golden paintbrush can be saved from extinction. This protocol for the micropropagation of golden paintbrush is directly from preformed meristem (axillary buds, shoot tips), without callus induction, which helps to maintain very low variability among the population of the same clone (Skirvin et al. 1994). Clonal population is desired for reintroduction of endangered plant species. Simple growing condition with minimal use of plant growth regulators has been found helpful to avoid somaclonal variation (Edson et al. 1996; Sarasan et al. 2006). Moreover, initiation of fresh culture from ex vitro mother plants growing in the field or greenhouse instead of keeping in vitro culture for longer time has been effective in minimizing somaclonal variation (Skirvin et al. 1996).
Reintroduction of endangered species has become increasingly important in conservation worldwide for recovery of rare species and restoration purposes (Maunder, 1992; Dunwiddie and Martin 2016). The basic biological purpose of reintroduction is establishing new or augmenting existing populations in order to increase a species’ survival prospects (Godefroid et al., 2011). Unfortunately, the reintroduction of rare and endangered plants has been very challenging due to low rates of success (Ren et al., 2012) as many endangered and rare species fail to reproduce in their natural habitat. For example, out of 45 reintroduction projects initiated in California, only 4 were successful (Allen, 1994).

In conclusion, an efficient in vitro propagation protocol was developed for golden paintbrush (*C. levisecta*), an endangered plant in Canada and USA. The protocol was developed for the induction of multiple shoot using nodal segments with high frequency ex-vitro rooting under greenhouse conditions. As a step towards in situ conservation, plantlets were re-introduced in their natural habitat. The micropropagation system offer an excellent opportunity for long term in situ conservation programs while also supporting ex-situ efforts through living plant collections and potential cryopreservation. To the best of our knowledge, this is the first report on micropropagation and re-introduction of *C. levisecta*, an endangered plant species in Canada.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Table 1. Effect of different concentrations of BA and Kn supplementation to Murashige and Skoog basal (MS) medium on the number of nodes and plant height produced by nodal explants of clone G2.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average no. of nodes per shoot</th>
<th>Average Plant Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA (µM)</td>
<td>Kn (µM)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>4.0</td>
<td>4.7 ± 0.35b</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>6.0 ± 0.33a</td>
</tr>
<tr>
<td>3.0</td>
<td>2.0</td>
<td>4.6 ± 0.38b</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>5.2 ± 0.34ab</td>
</tr>
</tbody>
</table>

**Note:** Data represents mean ± SE from about thirty-six explants per treatment and three replications. Means with the same letter in the graphs are not significantly different at $\alpha = 0.05$. 

https://mc.manuscriptcentral.com/cjps-pubs
**Fig. 1:** In vitro propagation of Golden paintbrush (*C. levisecta*). Seed pods and seeds used for in vitro germination (A). Seed germinated on semi-solid medium without growth regulators after four weeks (B). Nodal segments cultured on MS medium supplemented with 2.0 µM BA and 3.0 µM KN for shoot growth and development (C and D). Microshoots were transferred for root induction with 10.0 µM IBA treatment and in vitro rooting (E and F). Ex vitro rooting of microshoot immersed in 0.8% IBA rooting powder (G). *C. levisecta* plants acclimatized in the greenhouse (H and I) and were successfully reintroduced back to their natural habitat near Fort Rodd Hill, BC, Canada (J).

**Fig. 2:** Effect of different concentrations of BA and TDZ supplementation to Murashige and Skoog basal (MS) medium on the number of nodes produced by nodal explants of clone G1 and G2 compared to control (SDM, shoot development medium 2µM BA + 3µM Kn). Data represent mean ± standard error from about thirty-six explants per treatment and three replications. Means with the same letter in the graphs are not significantly different at α = 0.05.

**Fig. 3:** Effect of three different treatments with rooting powders (0.1, 0.4 and 0.8 % IBA) on root length and number of microshoots for clone G1 compared to control treatment for ex vitro rooting. Data represent mean ± standard error from about thirty shoots per treatment and three replications. Means with the same letter in the graphs are not significantly different at α = 0.05.
The diagram above shows the average number of roots per shoot and the average root length (mm) for different treatments.

- **Control**: Lower average number of roots per shoot compared to treatments.
- **Rooting 1**, **Rooting 2**, and **Rooting 3**: Higher average number of roots per shoot compared to Control.
- **Rooting 2** and **Rooting 3** have similar average root lengths, while **Rooting 1** has a slightly shorter average root length.

The diagram clearly indicates that treatments contribute to increased root growth compared to the control.