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Investigation into the effects of 6-Benzylaminopurine and 1-Naphthaleneacetic Acid concentrations on 3 micropropagated Begonia rex ‘Fedor’ explants

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ABSTRACT
Developing standardised tissue culture techniques for Begonia cultivars would assist botanical organisations in maintaining collections and enable sharing of plants worldwide. Currently there are no published papers on Begonia rex ‘Fedor’ regeneration in tissue culture. This project investigates the effects of 1-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BA) on three B. rex ‘Fedor’ explant types (lamina mid rib, petiole and leaf lamina) In Vitro. Relevant tissue cultures were placed in four varying concentrations of combined NAA and BA (0.70 µM and 0.60 µM, 0.55 µM and 0.98 µM, 0.20 µM and 0.26 µM, 0.28 µM and 0.49 µM) in Murashige and Skoog (1962) (Murashige and Skoog, 1962) medium. Explants were placed in optimum conditions (23.0°C ± 1.5°C with photoperiod of 16-hour light to 8-hour dark) for eight weeks and periodically monitored for root and vegetative expansion. The recorded data included multiple variables, therefore, a two-way ANOVA statistical test with replication was applied. Results found that leaf lamina explants placed in NAA 0.55 µM and BA 0.98 µM had the highest instance of leaf regeneration (2.7 per plant). The longest mean root length (2.24 cm) was observed in explants placed in 0.28 µM NAA and 0.49 µM BA. The lamina mid rib explants placed in 0.7 µM NAA and 0.6 µM BA gained the highest amount of fresh weight (0.52 g). The longest vegetative expansion was leaf lamina explants placed in 0.7 µM NAA and 0.6 µM BA. Further investigation for this project could include trialling other plant organs, such as the apical tip. Future re-trials could confirm that results are representative and accurate.

Keywords: Begonia, In Vitro Culture, Tissue Culture, NAA, BA

1. Introduction
This investigation aimed to identify a viable nutrient medium that supported the initial stages of explant regeneration of Begonia rex ‘Fedor’ for the efficient production of plants over a reduced time-period. Begonia produce very small seeds e.g. Begonia tribensis (280 µm length x 155 µm breadth) and Begonia rubella (284 µm length x 210 µm breadth) (Rajbhandary and Shrestha, 2010) which are notoriously difficult to work with and germinate, resulting
in commercial industries utilising vegetative propagation (often by lamina and terminal cuttings) to produce plants (Larson, 2012). Although a well-practiced technique, vegetative propagation can generate plant material that is vulnerable to contaminants: Begonia spp. is known to be susceptible to various pathogens such as Pythium, Rhizoctonia, Xanthomonas begoniae, Botrytis, Erysiphe and Phyllosticta (Digat and Vidalie, 1975). Identifying viable nutrient cultures which support Begonia cultivars In Vitro through to acclimatisation stages would be a useful tool to help maintain Begonia numbers within botanical collections, as well as enabling the sharing of disease-free plants with other organisations internationally (Bowes and Curtis, 1991). Begonia rex ‘Fedor’ is an ornamental foliage plant displaying distinctive silver foliage with near-green background colouration and contrasting (almost black) veins. The leaves are asymmetrical deltoid in shape. It was noted in the patent that the plant had not yet flowered, so a description of inflorescence could not be given (Hoefnagels, 2011). No published information concerning B. rex ‘Fedor’ could be found other than the 2011 patent. The lack of literature on this cultivar suggests no investigations into micropropagation techniques have taken place previously, indicating that experimental research into B. rex ‘Fedor’ and its response to tissue culture is unique.

2. Materials and methods

2.1 Nutrient Culture Composition

Concentrations of plant hormones are unique for each plant, even plants within the same genus and species can have different requirements and may respond in dissimilarly on the same media (Caponetti and Trigiano, 2011) There is no standardised medium for the micropropagation of B. rex ‘Fedor’, therefore, average 1-Naphthaleneacetic Acid (NAA) and 6-Benzylaminopurine (BA) concentrations for the control (C) were selected using the mean results of three relevant papers (Espino et al., 2004; Kaviani et al., 2015; Sara et al., 2012) which carried out similar research on micropropagated Begonia rex cultivars. Results selected for use were those showing maximum shoot regeneration and root extension in response to auxin and cytokinin concentrations. Using the mean optimum results from each investigation, it was established the C would contain 0.7 µM NAA and 0.6 µM BA (Table I).

Table I: Composition of four media in trial (basic media and NAA / BA concentration)

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>C</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS (g/l)</td>
<td>4.62</td>
<td>4.62</td>
<td>4.62</td>
<td>4.62</td>
</tr>
<tr>
<td>Sucrose (g/l)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Agar (g/l)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Dist. Water (ml)</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>NAA (µM)</td>
<td>0.70</td>
<td>0.55</td>
<td>0.20</td>
<td>0.28</td>
</tr>
<tr>
<td>BA (µM)</td>
<td>0.60</td>
<td>0.98</td>
<td>0.26</td>
<td>0.49</td>
</tr>
</tbody>
</table>
To establish NAA and BA concentrations for the three treatments studied (M1, M2 and M3), the research papers utilised for C media were referred to (Espino et al., 2004; Kaviani et al., 2015; Sara et al., 2012), as well as three investigative papers on tissue culture focusing on three different Begonia species (B. × tuberhybrida Voss, B. elatior and B. semperflorens) regeneration rates in response to NAA and BA concentration (Espino et al., 2004; Mendi et al., 2009; Nakano et al., 1999). Results obtaining the highest regeneration rates were selected for use and means of results used to decide concentration levels.

Basic nutrient media culture for Begonia contained 30 g/l sucrose, 8 g/l agar, 4.62 g/l Murashige and Skoog (1962) (Murashige and Skoog, 1962) (MS) and approximately 1000 ml distilled water (Scoggins and Bridgen, 2013) All media were comprised of identical base ingredients with only NAA and BA levels differing. The pH of nutrient culture media for Begonia was adjusted to 5.8 using NaOH or HCl prior to auto-claving (Espino et al., 2004)

### 2.2 Plant materials and surface sterilization

The B. rex ‘Fedor’ mother plant was obtained from a private collection at approximately one and a half years old, then monitored for three months prior to trial in an isolated indoor environment under average conditions of 15 - 30°C temperature and direct exposure to sunlight with weekly irrigation of 200 - 400 ml spring water.

The three explants selected for use in this trial were lamina mid rib (LMR), petiole (P) and leaf lamina (LL) (Fig 1).

![Fig. 1 Location of explants on plant: Circle: petiole, Square: lamina mid rib, Triangle: leaf lamina (Rowe, 2016)](image-url)
Leaf explant sizes were 0.25 cm$^2$ and petiole explants 2.0 mm cross-sections (Espino et al., 2004; Torres, 2012). The average fresh weight of explants at start were: LMR 0.011 g, P 0.063 g and LL 0.006 g.

Standard tissue culture techniques were used for obtaining explants and placing them into nutrient cultures (Cameron, 2013; Gamborg, 2013; George et al., 2008; Torres, 2012). An explant sterilising solution of 15% sodium hypochloride / 85% distilled water was used (ten minutes’ submersion), followed by three separate (two minutes and manually-agitated with tweezers) submersions in distilled water.

2.3 Trial size and In Vitro conditions

There were a total of 120 sterile sample bottles (30ml, Solas Pharma, LLC) in the investigation, comprised of four media combinations with ten of each explant type in each media (Table II).

Table II: Media type and corresponding explants

<table>
<thead>
<tr>
<th>MEDIA TYPE</th>
<th>EXPLANT TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (x30 TUBS)</td>
<td>LMR (x 10)</td>
</tr>
<tr>
<td></td>
<td>P (x 10)</td>
</tr>
<tr>
<td></td>
<td>LL (x 10)</td>
</tr>
<tr>
<td>M1 (x30 TUBS)</td>
<td>LMR (x 10)</td>
</tr>
<tr>
<td></td>
<td>P (x 10)</td>
</tr>
<tr>
<td></td>
<td>LL (x 10)</td>
</tr>
<tr>
<td>M2 (x30 TUBS)</td>
<td>LMR (x 10)</td>
</tr>
<tr>
<td></td>
<td>P (x 10)</td>
</tr>
<tr>
<td></td>
<td>LL (x 10)</td>
</tr>
<tr>
<td>M3 (30 TUBS)</td>
<td>LMR (x 10)</td>
</tr>
<tr>
<td></td>
<td>P (x 10)</td>
</tr>
<tr>
<td></td>
<td>LL (x 10)</td>
</tr>
</tbody>
</table>

Explants were stored in a Panasonic® MIR-154 cooled incubator (1.0, 15 W interior fluorescent light) with illumination kit MIR-L15-PE under optimum conditions of 23.0 °C ± 1.5 °C with a photoperiod of sixteen hours light and eight hours dark (Espino et al., 2004; Smith, 2013). Interval quantitative data was recorded on a weekly basis throughout the trial period of eight weeks on regeneration and changes in explant morphology (Phillips et al, 2013), with in-depth recording of root length, number of leaves, fresh weight and vegetative length occurring at the end of the trial at week eight.

2.4 Analysis

Microsoft Excel® 2016 was used to compile, analyse and produce figures from raw data collected. Due to the nature of the data, it was deemed suitable to apply a two-way analysis of variance (ANOVA) with replication. The significance level for this statistical tool was set at 0.05.
3. RESULTS

3.1 Contamination

Contamination resulted in a total of 20 out of 120 explants being removed (16.6%). Contamination was not equally distributed with C suffering the highest loss (eight in total: 26.6% of all C media). M3 showed the lowest contamination rate with three containers being withdrawn (10% of total M3). A significant difference between the week of the trial and type of explant excluded was found (P = 0.0008, < 0.05). The occurrence of contamination varied between media, explant type and week of the trial. The explants removed were six LMR, nine P and five LL. A significant difference was found between these variables (P = 0.00006, <0.05).

3.2 Root length

The longest average root length observed at week eight was the explants in M3 at 2.24 cm with the LL showing the longest roots at 2.62 cm (Fig. 2).

![Root Length Graph](image)

Fig. 2 Explant root length in M3. A significant difference was found between explant type and growth (P = 0.002, < 0.05).

The C explants had the shortest mean root length at 1.69 cm and the total average root length for all media and explants was 2.01 cm at week eight. A significant difference between explant and media type was found (P = 0.007, < 0.05).

3.3 Number of leaves

The total average leaves regenerated at week eight for all media and explants was 1.48 leaves per plant. A very significant difference was found between media and explant type (P = 0.000000003, < 0.05).
The highest overall average leaf regeneration was observed for explants placed in M1 (0.55 µM NAA and 0.98 µM BA) at 2.33 leaves per plant. Leaf lamina explants placed in M1 had the highest instance of leaf regeneration at 2.8 per plant at the end of the trial (Fig. 3).

![Graph showing leaf regeneration over weeks](image)

Fig. 3  Explant leaf regeneration in M1. No significant difference was found between explant type and number of leaves produced (P = 0.666, > 0.05).

### 3.4 Fresh weight

M3 (LMR) gained the most grams of fresh weight throughout the trial (0.90) (Fig 4), followed by C (LMR) at 0.52 g.

![Graph showing fresh weight](image)

Fig. 4  M3 explants start and end of trial fresh weight. Error bars show standard deviation. No significant difference was found between explant type and fresh weight gained for M3 (P = 0.20, > 0.05)
The media with the highest mean of fresh weight gained was M3 at 0.58 g. The lowest overall mean weight was seen in M2 (0.30 g). LMR explants gained an average of 0.53 g (the highest of explants) and P the lowest (0.31 g).

### 3.5 Vegetative length

At the end of week eight, eleven out of the twelve explant and media combinations had lengthened vegetatively. M2 (LMR) did not show any extension. The average growth for all explants in C was 0.46 cm, M1: 0.45, M2: 0.27 and M3: 0.24. The explant which had the longest growth in length was C (LL), with a total average of 0.74 cm followed by M1 (LMR) with 0.61 cm. The shortest growth was C (P) with 0.13 cm (Fig. 4).

![Vegetative Expansion](image)

Fig. 5  Vegetative expansion in cm for all media and explant combinations. Error bars show standard deviation. No significant difference was found ($P = 0.441, > 0.05$).

### 3.6 Mother plant inflorescence

The patent for *Begonia rex* ‘Fedor’ states that flowering had not been observed to date (2011) (Hoefnagels, 2011). While the main trial was underway, it was observed that the mother plant was flowering. Inflorescence are on raised single stems, flower type is single, approximately 1 - 2 cm in diameter with two pairs of opposite pale pink tepals (petal size is unequal symmetrically) with lower ones appearing larger. The stigma is compact, twisted, and bright yellow in colour. The inflorescence of *Begonia* are predominately monoecious (Agren and Schemske, 1991) (Fig 6).
4. DISCUSSION

All four of the experimental media showed signs of supporting the primary phases of establishment and regeneration to varying degrees of efficiency. Analysis of data obtained at week eight showed that three media (C, M1 and M3) and two explants (LL and LMR) had potential in regeneration and reduced contamination (Table 3).

<table>
<thead>
<tr>
<th>Area examined</th>
<th>Media with most potential</th>
<th>Explant with most potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowest contamination</td>
<td>M3</td>
<td>LL</td>
</tr>
<tr>
<td>Longest roots</td>
<td>M3</td>
<td>LL</td>
</tr>
<tr>
<td>Highest number of leaves</td>
<td>M1</td>
<td>LL</td>
</tr>
<tr>
<td>Length of vegetative section</td>
<td>C</td>
<td>LL</td>
</tr>
<tr>
<td>Fresh weight</td>
<td>C</td>
<td>LMR</td>
</tr>
</tbody>
</table>

4.1 Contamination

The disparity with distribution of contamination between media indicates that there may have been a specific instance of contamination when the C media was being prepared or paired with its respective explant. There are multiple microscopic organisms responsible for contamination in tissue culture, including: bacteria, yeasts, filamentous-fungi, viruses, viroids and micro-arthropods. Incubation and multiplication periods prior to expression varies between species making identification of contaminant sources difficult (Leifert and Cassells, 2001). The two most common contaminants to affect tissue culture and bacterial and fungal based, these microbes are present in the
atmosphere in vast quantities, therefore, if pathogens came into contact with the explant or tissue culture during preparation stages, they will take advantage of the optimal growing conditions provided and flourish. Fragile plant tissue placed in this environment will consequently become infected and die (Razdan, 2003).

Alternatively, the levels of growth hormones within the media could be inadequate as a tissue culture medium, resulting in the primary stages of explant initiation being retarded and consequentially the plant cellular tissue dying and initiating decomposition. If the selection of phytohormones and concentrations were unbalanced for this cultivar it can have an effect on the formation of organs (Torres, 2012).

In a study by Takanka et al. (2012) (Tanaka et al., 2012), Begonia x tuberhybrida, explants were examined for adventitious bud formation. At eight weeks the contamination rate varied between 7 – 87% due to contamination or withdrawal due to explant death. Comparatively, the 16.6% contamination rate of this trial is lower, indicating NAA and BA levels are not incompatible with growth.

### 4.2 Roots

The longest average root length in the trial was observed for the explants in M3 and was 2.24 cm, the shortest average was the C explants at 1.70 cm (a difference of 0.54 cm root length). Comparatively, in similar research into Begonia tissue culture, Gergely and Cachita-Cosma (2011) (Gergely and Cachita-Cosma, 2011), found that when Begonia semperflorens ‘Ambassador’ explants had been cultured for 30 days in nutrient medium containing 2.27 µM Thidiazuron (TDZ) and 2.46 µM Indole-3-butyric Acid (IBA) the average length of the longest root was 1.02 cm. At the same stage in this trial (between weeks four to five) the average longest root was 1.27 – 1.36 cm for the C media, M1: 1.29 – 1.35, M2: 1.56 – 1.71 and M3: 1.55 – 1.73. The overall average for all explant types and media combinations at 28 to 35 days was 1.42 – 1.54 cm.

It was observed that the lowest root length average at week eight was C (P) at 1.4 cm, however, the LMR (1.62 average) and LL (2.02 average) also cultured in C performed within expected ranges: the overall mean root length for all media was 2.01 cm, meaning the LL was 0.01 cm over the trial average. Although LMR was below the total trial average by 0.39 cm, there were two other explants (M1 (LL) and M2 (LL) within 0.03 of this length.

A possible explanation for C (P) having comparatively shorter roots is that only five of the total ten explants from the start the trail remained at the end; the other five being withdrawn due to contamination. As there may have been an underlying contamination issue (compared to the other explants / media) with the petiole explants from C, it could be purported that there may have been underlying contamination with the remaining explants which resulted in retarded root extension. Cytokinin may have an inhibiting effect on root development by restricting the inception of primordium roots (Aloni et al., 2006; Fukaki et al., 2002). The apical dominance of roots is regulated by cytokinin present in the root tip calyptra which prioritises the establishment of primary roots over lateral (Aloni et al., 2006).
4.3 Leaves

All of the media types were capable of supporting explants that produced leaves. The highest number of leaves generated was seen in the explants placed in M1 with an average of 2.33 leaves per plant and the lowest the M2 media at 0.6. M3 and C had similar foliage production at 1.4 and 1.6 per plant respectively. For M1 the explant with the highest leaf regeneration was the LL at an average of 2.7 per plant, the second highest within the C media was the LMR at 2.3. The P was the lowest at 2 leaves per plant. In Begonia plants placed in tissue culture, it has been noted that explants procured from the lamina areas in particular are especially prolific at regenerating shoots and produce higher numbers compared to other plant regions (Razdan, 2003). The least productive media for leaf growth was M2 with a total average of 0.6 leaves per plant produced. The explants had varying production rates (LMR: 0, P: 1.4 and LL: 0.4). It may be worth noting that although M2 had the lowest number of average leaves p/plant it did have the longest average root length out of the trial.

4.4 Fresh Weight

All explant types in the four media gained weight over the duration of the trial. The explants that had gained the most weight at week eight were the LMR in M3, gaining 0.90 g.

Callusing is a natural response to wounding of plant organs: the aim is to repair affected areas by inducing division of adjacent unaffected cells, creating a protective seal over the location of damage comprised of lignin, suberin and wax deposits. If initial wounding processes are immediately succeeded by aseptic conditions (*In Vitro*), cellular division responses can become over-stimulated indefinitely as a result of nutrients, minerals and phytohormones present in the culture (Hall, 2013). Though not evenly distributed throughout media and explant type, it was observed that extreme callusing build-up was present on some explants with the highest concentration on the M3 (LMR). Root formation was present, however, leaves were not in most extreme cases (Fig. 7).

Fig. 7  Example of abnormal callusing in M3 (LMR) (Rowe, 2016)
Additionally, M3 (LMR) had the shortest overall root length (1.79 cm) and leaf number (1.1). Callusing varied in diameter from approximately 0.1 – 0.6 cm length and 0.1 – 0.5 cm in height with a crystallised white, yellow and light green coloured appearance. The reason for M3 (LMR) weighing more than all other explants on average could be a result of excessive callus formation due to trauma induced by micropropagation processes. For this reasoning, it is deemed that the weight of M3 (LMR) is not a reliable example of normal growth regarding fresh weight. The next highest fresh weight observed was in C (LMR) which rose from 0.011 g to 0.53 g (0.52 g). This explant had a root extension of 2.14 cm and 1.8 leaves p/plant which provides a plausible reason for weight being above average compared to the other plants in the trial. The lowest average of fresh weight gained was the LL explants in M2, they gained 0.26 g (0.006 g to 0.27 g), these explants had an average root length of 1.65 cm (0.36 cm below the overall trial average of 2.01 cm) and an average of 0.4 leaves (1.08 leaves below the trial average of 1.48 leaves p/plant). As the rooting and leaf production was below average it is concluded that this is the reason for gained weight being lower than other explants.

4.5 Vegetative Length

Eleven out of the total twelve combinations of media and explant type had increased in length vegetatively (from the bottom of the basal section to top of vegetative stem at highest point). The optimal result seen in the media / explant combination of C (LL) (0.74 cm) may be explained by the high auxin (NAA) levels in the nutrient culture. The NAA for the C media was the highest out of the four media trailed at 0.70 µM, the NAA rates for the other media varied between 0.20 – 0.55 µM. Auxin is an organic acid (NAA a synthetic version) that influences cellular division and mitosis and particularly associated with elongation and differentiation. The compound is partially responsible for eventual physical structure and function of cell tissue in higher plants (Ljung, 2013). As auxins are integral to plant elongation and C (LL) media had the highest concentration of the trial, it could be suggested that the reasoning for the longest vegetative extension is a result of high NAA levels within the nutrient media. The shortest vegetative length was measured in C (P) (0.13 cm). The P explants in media C experienced issues with high contamination rates and possibly retarded growth as a result of potential unidentified infection. For this reason, it is recommended that the C (P) lowest vegetative measurement may be unreliable as a normal example of growth. The second lowest average of elongation was observed in M1 (P) and third lowest in M3 (P). As all three of the lowest extensions are seen in P explants it is implied that P explants are potentially not viable explant types for optimal growth.

5. CONCLUSION

Overall, this study was fruitful in achieving the aim of identifying a viable nutrient culture for the regeneration of B. rex ‘Fedor’ and giving a deeper insight to an area of
research not fully explored. Three viable media (C, M1 and M3), capable of supporting LMR and LL explants through the initial establishment and regeneration stages were identified. In order for the media with positive traits to be utilised commercially or by botanical organisations, future re-trials and honing of the plant hormones to find an optimal balance would need to take place. A future trial focusing on NAA and BA concentrations based on the limits identified as productive for tissue culture of B. rex ‘Fedor’ in this research (NAA: 0.28–0.70 µM and BA: 0.49–0.98 µM), would be beneficial to identify the optimum auxin and cytokinin requirements. The explant type to be placed In Vitro should include LMR and LL as both showed good response in regeneration. P explants should not be considered for inclusion in future research, however, other plant organs such as the apical tip could: due to it being identified as very successful in propagating (George et al., 2007).

A more focussed and larger trial (with re-trial) may extract more concise data on identifying efficient tissue culture technique for B. rex ‘Fedor’. It is hoped that the findings from this trial will contribute specifically to the knowledge of B. rex ‘Fedor’ and add to the pool of information already known on other B. rex cultivars.

REFERENCES


