Evaluating the Effect of Photomixotrophic Conditions for Expedite and Efficient In Vitro Tuberization System on the Morphological, Biochemical, Anatomical and Physiological Characteristics of Microtubers

Siddra Ijaz 1, Erum Shah 2, and Imran-ul-Haq 3

1 Centre of Agricultural Biochemistry and Biotechnology/US-Pak Center for Advanced Studies in Agriculture and Food Security, University of Agriculture Faisalabad, Pakistan
2 Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture Faisalabad, Pakistan
3 Department of Plant Pathology, University of Agriculture Faisalabad, Pakistan

* Corresponding email: siddraijazkhan@yahoo.com


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Abstract In vitro tuberization is becoming a swift approach to expedite seed tuber production and multiplication for various purposes such as germplasm conservation as well as exchange, experimentation in basic research, in vivo selection of desirable agronomic traits and disease free tuber production. Therefore, this study was designed for optimizing in vitro tuberization system using different photomixotrophic conditions in tissue culture regime. For this purpose, sucrose, BAP and kinetin were used at different levels and combinations for evaluating their effects on proficient and expedite in vitro tuberization system in selected local potato cultivars, PRI-Red. The results showed that tuberization medium, TM5 (100 g/l sucrose; 4.75 mg/l kinetin but deprived of BAP) proved to be best in terms of maximum number of microtubers per explant achieved. Subsequently, morphological, anatomical, physiological and biochemical characteristics of these in vitro grown microtubers were studied for evaluating the effects of photomixotrophic conditions on the said characteristics of microtubers.

Keywords Potato; Tissue culture; Potato anatomy; Proximate analysis; Photomixotrophic condition

1 Introduction
Potato is the fourth most important food crop, after wheat, rice and maize. This crop is grown under a large area all over the world and is being used as a staple food in developing countries like Pakistan, Bangladesh and India. In our agro-climatic conditions, this crop is generally grown vegetatively through tubers. For vegetative reproduction, tuber is used as a seed. Whereas in conventional breeding system, crosses are made that are followed by the selection and screening of subsequent progenies possessing desired traits. These selected F1 material is then clonally or vegetatively propagated that result in the fixing of these required traits (Sleper and Poehlman, 2006). However the F1 heterozygous material gives heterotic effect results in hybrid vigor while on selfing it leads to inbreeding depression (Arndt and Peloquin, 1990). Hence, in conventional seed potato production the risk of viral, bacterial and fungal diseases is increased with every round of progeny multiplication in the field.

The total cost on potato seed is very high, because just cost on seed transportation and storage is about 47% of the total cost. Though best quality potato seed is generally imported from other countries that results in huge economic burden. Therefore in this crop, different strategies are being adopted for seed production either via conventional means or non-conventionally. Among these various strategies and techniques for seed production in potato, in vitro microtuberization holds numerous intrinsic worth in terms of ease in transportation, handling and long term storage. Whereas potato tuberization is very complex developmental process which is regulated by many factors (Altimadal and Karadogan, 2010). Tuber growth and developmental processes are very difficult to study in field grown plants because of obscuring effect of soil and little synchronization of the tuberization
process but \textit{in vitro} system provides the detailed description of the whole phenomenon. Thus microtuber production is one of the strategies under this perspective.

In Potato, \textit{in vitro} tuberization is a highly complex phenomenon that can be induced under \textit{in vitro} conditions. Because of their small size and weight, microtubers have remarkable benefits for getting disease free plant material, easy storage and transportation (Kefi et al., 2000; Kanwal et al., 2006) over conventionally grown seed potatoes. The other benefits are the less chance of drying out while storage, short dormancy time and high rate of survival in direct transfer to soil (Kefi et al., 2000). Therefore different research groups around the globe are trying to bring about this revolution (Gopal et al., 2004; Zhijun et al., 2005; Zhang, 2006). Various evidences for resilient and consistent analogies between \textit{in vitro} and field grown tubers for their induction, growth and development have been documented in literature.

There are several components such as the rapid and near synchronous induction and growth that can be modified by using a range of exogenous compounds and even altering growth conditions, make the microtuber a valuable model system (Coleman et al., 2001). This technique reduces the time taken to yield seed tubers, lessen the number of field generations needed and ultimately in higher class seed tubers (Jones, 1994). Keeping in view the above mentioned benefits of \textit{in vitro} tuberization approach, this study was designed. In this research study, photomixotrophic conditions for a proficient and expedite \textit{in vitro} tuberization system in selected local genotype of potato (cv. PRI-Red) was optimized. In addition to this, photomixotrophic conditions in terms of their effect on morphological, anatomical, physiological and biochemical characteristics of microtubers were evaluated.

2 Material and Methods

\textit{Potato genotype to be used:} Local Pakistani Potato cultivar, PRI-Red was collected from Potato Research Institute, Sahiwal, Pakistan.

\textit{Explant to be used:} \textit{In vitro} micropropagated shoot was used as explant.

\textit{Culture media for \textit{in vitro} tuberization:} Nine different media were formulated for \textit{in vitro} tuberization of cv. PRI-Red. Each medium contains basal MS salt and vitamins, supplemented with 60 g/L, 80 g/L and 100 g/L sucrose; 0, 3.75, 4.75, 4.5 mg/L, 5 mg/l Kinetin and 0, 3.75, 4.75, 5.5 mg/l, 6 mg/l BAP, alone or in combination. These media formulations were referred as; TM1, TM2, TM3, TM4, MPm5, MPm6, TM7 and TM8 and TM9 (details of these media is given in Table 1). All media components were mixed together, adjusted to pH 5.7-5.8 and solidified with 2.66 g/l Gellan gum powder. Thereafter, these media were autoclaved at 121°C and 15 psi for 20 minutes.

| Table 1 Tuberization media (TM): MSN* (MS salt = 4.33 g/l, Myoinositol = 0.1 g/l, Nicotinic acid = 1 g/l, Pyridoxine HCl = 1 g/l, Thymine HCl = 2 g/l, Glycine = 4 g/l, Gellan gum powder = 2.66 g/l) |
|---|---|---|---|---|---|---|---|---|---|
| TM1 | TM2 | TM3 | TM4 | TM5 | TM6 | TM7 | TM8 | TM9 |
| Sucrose | 60 g/L | 100 g/L | 80 g/L | 80 g/L | 100 g/L | 80 g/L | 80 g/L | 80 g/L |
| Kinetin | 0 mg/l | 0 mg/l | 3.75 mg/L | 4.75 mg/L | 4.75 mg/L | 0 mg/l | 0 mg/l | 4.5 mg/L |
| BAP | 0 mg/l | 0 mg/l | 0 mg/l | 0 mg/l | 0 mg/l | 3.75 mg/L | 4.75 mg/L | 5.5 mg/L |
| Nutrient spp. | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* |

\textit{In vitro} tuberization: \textit{In vitro} micropropagated shoots of selected genotypes (cv. PRI-Red) were cultured on all nine \textit{in vitro} tuberization media describe. For culturing, the micropropagated shoots were excised in such a manner that each cutting contained 1-2 buds. All operations were accomplished in sterile conditions, under laminar air flow hood. Cultures were maintained in dark conditions at 21°C as well as some cultures were kept at 26±1°C for 16/8 hrs light/dark regimes. Subsequent maintenance of these cultures was achieved by biweekly subcultures.
Experimental layout and statistical analysis: Experiment was repeated three times and in each repeats 10 test tubes and each containing single explant were used. The experiment will be conducted in completely randomized design (CRD). Analysis of variance (ANOVA) will be constructed and All-pair wise comparison test will be calculated at 5% probability level among various treatments (Steel and Torrie, 1986).

Data recording: Data were collected in the form of stolon length, number of days for the initiation of microtuberization, number of microtubers produced per explant, time period for microtubers maturation, skin color, flesh color, proximate analysis, effect of physiological age of microtubers on sucrose and starch contents and histoanatomical comparison between microtubers and wild type potato.

Histoanatomy: As mentioned earlier, in this research study in vitro microtuberization in studied potato genotype was tried to be established using different photomixotrophic conditions. As the results showed that excellent in vitro microtuberization was achieved in TM5. Hence for histoanatomy, microtubers of TM5 were used and compared with field grown potato tuber of same genotype. For this, thin sectioning of both microtubers and field grown tubers were performed to prepare slides for microscopy. Staining of tissue was done using Safranin dye and Fast green dye. After that tissue was fixed by latex and covered with cover slip.

Effect of physiological age on sucrose and starch contents: The effect of physiological age of tuber on sucrose and starch contents was also investigated. For this, a comparison was made between microtubers of two different ages viz., 35 days old and 60 days old. For determining the sucrose and starch contents in microtubers of both age groups, microtubers were dried in oven at 60°C for 48 hours and subsequently were ground into fine powder. Then, for preparing sucrose solution 20 mg of this powder were added in 800μl d2H2O and then vortex briefly followed by centrifugation at 13200 rpm for 5 minutes. Supernatant was taken into fresh reaction tube and then stored. Similar way was adopted for preparing starch solution but in this case sample was incubated at 70°C for 90 minutes prior to centrifugation. Then sucrose and starch contents were estimated using UV visible spectrophotometry. Sucrose and starch contents were quantified by recording the absorbance at 620 nm and 630 nm respectively.

Proximate analysis: Effect of photomixotrophic conditions on biochemical attributes was also investigated. For this purpose, proximate analysis of microtubers and field grown tubers of PRI-Red was performed. Thereby, dry matter, crude proteins, fat, fiber extract and ash contents were estimated. For dry matter, in vitro microtubers and field grown potato tubers were weighed before and after drying in an oven for 48 hours at 60 °C and ground into fine powder. Crude protein was estimated using Kjeldahl method, while fat was determined by soxhlet extraction and defat sample taken after fat extraction was used to determine fiber extract after removing protein and carbohydrates by treating it with H2SO4 and NaOH respectively. However ash content was measured by heating the sample. For this sample was taken in crucible and placed in pre-heated Muffle Furnace (at 350°C for 24 hours).

3 Results and Discussion
The effect of photomixotrophic conditions on in vitro microtuberization in terms of their effect on morphology, biochemistry, physiology and anatomy of microtubers was investigated. For this purpose, nine in vitro tuberization media having different photomixotrophic conditions were investigated and data was recorded in the form of number of microtubers per explant, stolon length, number of days of microtubers, skin color, flesh color, physiological, biochemical and anatomical characteristics. In vitro micropropagated shoots of potato cv.PRI-Red were cut axenically in laminar air flow hood in such a way that each cutting contained 1-2 buds. These cutting of genotype to be studied were cultured on media for investigating the response of these media to microtuberization. The data were recorded in the form of number of microtubers produced per explant. Subsequent to this, data collected were subjected to the statistical analysis. In this case, Analysis of Variance table (Table 2) revealed significant variation among in vitro tuberization media. All-pair wise comparison test depicts that, tuberization medium 5 (TM5) was excellent (Table 3; Figure 1) because maximum number of microtubers per explant was
achieved on this medium (Figure 2) followed by TM4 whereas TM1, TM3, TM7, TM8 and TM9 all showed poor response for *in vitro* tuberization (Table 3).

Table 2 Analysis of variance (ANOVA) table: ANOVA depicts the significant variation in response of photomixotrophic conditions of tuberization media on *in vitro* tuberization

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>SS</th>
<th>MSS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>8</td>
<td>22661.1</td>
<td>2832.63</td>
<td>822**</td>
<td>0.00</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>62.0</td>
<td>3.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>22723.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ** = Highly significant (P value <0.01)

Table 3 All-pairwise comparisons test of no. of microtubers from tuberization media to be studied

<table>
<thead>
<tr>
<th>Media</th>
<th>TM 5</th>
<th>TM 4</th>
<th>TM 2</th>
<th>TM 6</th>
<th>TM 1</th>
<th>TM 3</th>
<th>TM 7</th>
<th>TM 8</th>
<th>TM 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>81</td>
<td>60</td>
<td>37</td>
<td>25</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: α = 0.05; SE= 1.5154

Figure 1 A graphical representation of response of tuberization media, on average number of microtubers per explant

Figure 2 Response of photomixotrophic conditions of TM5 on *in vitro* tuberization

*Effect of light and dark regimes on *in vitro* microtuberization:* In this study effect of light and dark regimes were also investigated. For this purpose, cultured explants were kept in complete dark regime as well as in 16/8 hour light/dark regime. When explants were maintained in dark regime at 21± 1 °C, then microtubers were induced.
But the case was otherwise in case when explants were incubated at 26 ± 1°C under 16/8 hrs light/dark regimes. In this case, instead of microtubers formation, flowering was induced (Figure 3). In this study, transitions of cultured material from dark to light condition and vice versa were also done to examine the effect. Thus microtubers produced in dark conditions were when kept in light conditions for some time; they turned to greenish black in color (Figure 4). This might be due to the synthesis of chlorophyll. Similarly, when the cultured material that was initially kept in light/dark regime and they showed flowering, when kept in dark conditions they showed response towards microtuberization (Figure 5).

**Figure 3** Responses of *in vitro* microtuberization under 16/8 hrs light/darkregime

**Figure 4** Response after transferring culture tubes from dark to light regimes

**Figure 5** Response after transferring culture tubes from light to dark regimes

**Effect of photomixotrophic conditions on morphological parameters:** The responses of all tuberization media were also different regarding days to stolon induction, days to tuber initiation/induction, days to tuber maturation and stolon length (Table 4). Microtuber maturation data were recorded on the basis skin color. When skin color
was changed, from off white creamy to brownish then data were noted. In addition to this, another morphological parameter viz., Stolon length, flesh color, skin color, were also measured. In stolon length, solons from all media were taken and their height was measured and the height was ranging from 4 cm to 7 cm. While skin and flesh colors were off-white creamy in case of microtubers from all media to be studied.

Table 4 Response of photomixotrophic conditions of all tuberization media on invitro tuberization

<table>
<thead>
<tr>
<th>Number of days to stolon induction</th>
<th>TM 1</th>
<th>TM 2</th>
<th>TM 3</th>
<th>TM 4</th>
<th>TM 5</th>
<th>TM 6</th>
<th>TM 7</th>
<th>TM 8</th>
<th>TM 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average stolon length</td>
<td>6.2 cm</td>
<td>5.8 cm</td>
<td>4.3 cm</td>
<td>6.2 cm</td>
<td>7.9 cm</td>
<td>5.0 cm</td>
<td>3.7 cm</td>
<td>2.6 cm</td>
<td>1.3 cm</td>
</tr>
<tr>
<td>Number of days to microtuber induction</td>
<td>-----</td>
<td>5 days</td>
<td>-----</td>
<td>7 days</td>
<td>5 days</td>
<td>14 days</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Number of days to microtuber maturation</td>
<td>-----</td>
<td>33 days</td>
<td>-----</td>
<td>40</td>
<td>43</td>
<td>50 days</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
</tbody>
</table>

Effect of photomixotrophic conditions on histoanatomy: Effects of photomixotrophic conditions on histoanatomy of microtubers were also investigated. The anatomical structure of a typical potato is seen in microtubers as well as in field grown potatoes. The layer of epidermis encloses large mass of storage parenchyma, the cortical parenchyma, which is composed of large thin-walled cells, more or less regularly arranged. This whole region is covered with numerous starch granules in both cases. There is no clear difference in the anatomy of microtubers and field grown potatoes. But in microtubers, there has a small constricted area in central portion indicating the actively growing cells. The anatomical comparison showed that microtubers are closely related to the field grown potatoes. Both have same arrangement of cortical parenchymatous cells and the same storage cells for the deposition of starch granules. The only difference seen in microtubers is the presence of the actively growing cells in the central region (Figure 6; Figure 7).

Figure 6 Photomicrograph of invitro grown microtuber

Figure 7 Photomicrograph of field grown potato tuber

Effect of physiological age of microtubers on sucrose and starch contents: For determining the effect of physiological age on sucrose and starch contents spectrophotometry was done. In this experiment, sucrose-starch transition in different physiological ages of microtubers was estimated. For this purposes microtubers of 35 days as well as 60 days were selected. This experiment revealed that more amount of sucrose and less amount of starch were present in 35 days old microtubers whereas the case was otherwise in 60 days old microtubers. Thirty five
(35) days old microtubers contained 1.775 mg/dl sucrose and 0.9665 mg/dl starch while 60 days old microtubers contained 0.702 mg/dl sucrose while 1.4577 mg/dl starch (Table 5). These results depicted that sucrose is more in microtubers of less physiological age while at this stage starch content is low. Contrary to this, increase in physiological age resulting in low sucrose content but high starch content.

Table 5 Sucrose and starch contents in microtubers of different physiological age

<table>
<thead>
<tr>
<th>Age of microtubers</th>
<th>Sucrose contents</th>
<th>Starch contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 days</td>
<td>1.775 dl</td>
<td>0.9665 dl</td>
</tr>
<tr>
<td>60 days</td>
<td>0.702 dl</td>
<td>1.4577 dl</td>
</tr>
</tbody>
</table>

Note: *dl= decilitre

Effect of photomixotrophic conditions on biochemical characteristics of microtubers: For comparative biochemical analysis, proximate analysis was performed to estimate the chemical composition of the in vitro grown microtubers and field grown potato tuber. In proximate analysis, dry matter, crude protein, fiber extracts, fats and ash contents on dry basis were estimated. Thereby a comparative chart was made (Table 6) which depicted that dry matter, crude proteins and fiber extracts were more in microtubers as compared to field grown tuber except fat percentage that was observed as same in both cases. But ash percentage was more in field grown tubers as compared to microtubers.

Table 6 Proximate analysis of field grown potato tubers and in vitro grown microtubers

<table>
<thead>
<tr>
<th></th>
<th>Field grown potato tuber</th>
<th>In vitro grown Microtuber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>29 %</td>
<td>34 %</td>
</tr>
<tr>
<td>Crude protein</td>
<td>13.3 %</td>
<td>14.6 %</td>
</tr>
<tr>
<td>Fats</td>
<td>0.7 %</td>
<td>0.66 %</td>
</tr>
<tr>
<td>Fiber extract</td>
<td>1%</td>
<td>1.4 %</td>
</tr>
<tr>
<td>Ash content</td>
<td>7 %</td>
<td>6 %</td>
</tr>
</tbody>
</table>

As discussed already in this paper, in vitro micropropagated shoots of potato, cv. PRI-Red were used as explant, which were cultured on nine tuberization media having different photomixotrophic conditions. These media supplemented with basal MS salt in common contained sucrose, kinetin and BAP at varying concentrations and in different combination as well. While maximum number of microtubers per explant was achieved on medium, TM5 containing 100 g/l sucrose, 4.75 mg/l kinetin and no BAP. The results of this study revealed that sucrose has significant effect on microtuberization. In this study three levels of sucrose viz., 60 g/l, 80 g/l and 100 g/l were investigated and at sucrose concentration 60 g/l, no tuberization response was observed while medium (TM5) containing 100 g/l sucrose and 4.75 mg/l kinetin showed excellent response for microtuberization followed by medium (TM4) having 80 g/l sucrose and 4.75 mg/l kinetin. The importance of sucrose at increased level in tissue culture regime for efficient microtuberization was also documented by Yu et al. (2000); Shibli et al. (2001); Rida et al. (2001); Gopal et al. (2004). Similarly response of media containing kinetin was good rather than media having BAP. This showed that kinetin is more important for microtuberization as compared to BAP. Similar findings were reported by Gopal et al. (1998); Amma and Maity (1998) and Rodrigues-otubo et al. (1999).

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