Assessing the Effect of Naphthalene Acetic Acid and 6-Benzylaminopurine on In Vitro Micropropagation of Potato (Solanum tuberosum L.) and Estimation of Secondary Metabolites in In Vitro Micropropagated Shoots

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Abstract The effect of Naphthalene acetic acid (NAA) and 6-Benzylaminopurine (BAP) was investigated on in vitro micropropagation of potato (Solanum tuberosum L.) and secondary metabolites of in vitro micropropagated shoots were also estimated. Eight micropropagation media containing different level of NAA and BAP were studied and the effect of NAA and BAP on in vitro micropropagated shoots was assessed. However, in this research study, medium MPm 7 having 2.75 mg/l BAP was proved to be best for giving maximum number of shoots per explant in both genotypes to be studied. The genotype, PRI-Red yielded maximum number of shoots on this medium thus these in vitro micropropagated shoots were subjected to phytochemical analyses and were compared with shoots of intact plant (field grown) for secondary metabolites viz., alkaloids, flavonoid and total polyphenolic contents. In phytochemical analyses, no alkaloids were not detected in both in vitro micropropagated shoots and shoots of intact plant while both plants showed positive test for presence of polyphenolics. Shoots of intact plant contained higher amount of total polyphenolics (3.7 GAE mg/g fresh wt.) as compared to in vitro micropropagated shoots (2.8 GAE mg/g fresh wt.). However, slightly higher amount of flavonoid were detected in in vitro micropropagated shoots (1.8 mg/g) than shoots of intact plant (1.6 mg/g).

Keywords Potato; Polyphenoles; Micropropagation; 6-Benzylaminopurine; Naphthalene acetic acid

1 Introduction

Potato (Solanum tuberosum L.) is one of the most important vegetable crop, belongs to an economically important family Solanaceae and is grown worldwide. In some countries like Bangladesh, it secured second position as a staple food crop after rice (Parveen et al., 2014). Due to its high nutritious value, the potato crop has gained much importance. It is considered as the third most important crop of the world after wheat and rice and is consumed by more than one billion people worldwide (Venkatasalam et al., 2013). In Pakistan, it was grown on 172 thousand hectares during the year 2012 with the production of 3767.2 thousand tons which was 11.0 % more than last year (Economic survey of Pakistan, 2013). In Pakistan, the production rate of the potato crop is very low compared to other countries due to the poor-quality seed.

Conventionally the potato crop is propagated vegetatively by seed tubers which give uniform growth and yield. The problem of deterioration of crop is faced due to virus infection and degree of deterioration changes from one crop to other and also to cropping seasons. The transmission of viruses is due to the planting of infected tubers. The prevalence of virus infection can reach up to 100% in successive crop seasons and can reduce the yield up to half or one third. This is the major problem faced by seed tuber producers.

As discussed above the potato crop is usually propagated asexually by using tubers. The vegetative propagation in potato can also be achieved through tissue culture which is a technique of great potential in the field of
agriculture. In tissue culture techniques, especially the micropropagation technique is very important and effective technique which can produce thousands of vegetatively propagated plants in less time and by using less space. Micropropagation technique facilitates the production, multiplication and maintenance of disease free potato clones (Venkatasalam et al., 2013).

Potato crop is prone to stresses by different biotic and abiotic factors and among these; frost is a very important factor. Frost tolerant germplasm of potato crop was imported but now in Pakistan, different research institutes are working on this aspect. A number of frost tolerant genotypes have been approved. Hence, this proposed research study was aimed at the establishment and optimization of an efficient, expedite and genotype-independent micropropagation system in selected genotypes of potato to produce healthy, disease free material in short time and less space for the farmers. For this research study, axially buds of sprouts were used as explants. The explants were induced into plantlets on tissue culture regime containing different growth regulators at different levels and even in different combinations. However in this study effect of best micropropagation medium on secondary metabolites of in vitro micropropagated shoots was also assessed and estimated.

2 Material and Methods

Germplasm to be used: Two local genotypes of potato (Solanum tuberosum L.) viz., PRI-Red and FD 69-1 were used for this research study.

2.1 Explant preparation

a) Sprouting of Potato Tubers: For expedite sprouting, potatoes of both genotypes were incubated under dark and cold regimes. Potatoes were washed with ultra pure water and kept in cold condition at 4°C for 5 days and subsequent storage in dark condition at 26±1°C for one month.

b) Sterilization of Explants: Surface sterilization of explants (Axillary bud of sprout) was done by using different detergents and antiseptic solutions.

Shoots induction/in vitro micropropagation: Eight micropropagation media (Table 1) were used in this experiment. These media were investigated for efficient and genotype independent in vitro micropropagation system in Solanum tuberosum L. The explants were induced into plantlets on tissue culture regime containing NAA and BAP growth regulators at different levels and even in different combinations. For culturing, sprouts were cut in such a way that each cutting must contains 1 or 2 buds. All cultures for in vitro micropropagation were kept and maintained under 16 hours photoperiod at 26±1°C.

Table 1 Micropropagation media (MPm)

|          | MPm1 | MPm2 | MPm 3 | MPm 4 | MPm 5 | MPm 6 | MPm 7 | MPm 8 |
|----------|------|------|-------|-------|-------|-------|-------|-------|
| NAA      | 0 mg/l | 0.75 mg/l | 1.75 mg/l | 2.75 mg/l | 0 mg/l | 0 mg/l | 0 mg/l | 2.75 mg/l |
| BAP      | 0 mg/l | 0 mg/l | 0 mg/l | 0 mg/l | 0.75 mg/l | 1.75 mg/l | 2.75 mg/l | 2.75 mg/l |
| Nutrient spp. | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* | MSN* |

Note: MSN* (MS salt = 4.33 g/l, Sucrose = 30 g/l, Myoinositol = 0.1 g/l, Nicotinic acid = 1 g/l, Pyridoxine HCl = 1 g/l, Thymine HCl = 2 g/l, Glycine = 4g/l, Gellan gum powder = 2.66 g/l)

Rooting and acclimatization of micropropagated shoots: For the induction of healthy, strong and profuse rooting, micropropagated shoots were transferred to rooting medium containing basal MS salt (M524, PhytoTechnology Laboratories®, USA), 3% sucrose, vitamins, devoid of growth regulator (s) and 0.266 % Gellan gum powder for solidification of medium. Rooted shoots or plantlets were shifted to pots containing Belgium compost and wrapped with polythene bag to maintain humidifying condition at 26±1°C. These plantlets were then slowly acclimatized to lower humidity prior to green house transfer.

Experimental layout and statistical analysis: The data was collected as the number of shoots per explant and the days to growth initiation after culturing. The experiment was conducted in completely randomized design (CRD).
Analysis of variance (ANOVA) was constructed and Duncan’s multiple range test (DMRT) was calculated at 5% probability level among various treatments (Steel and Torrie, 1986).

2.2 Phytochemical analysis

Total Phenolic Contents: The total phenolic contents of shoots of intact plant and in vitro micropropagated shoots were determined by Folin-Ciocalteu method (Jahan et al., 2011). Gallic acid was used as standard to compare the results. Various concentrations (0.01 to 0.10 mg/ml) of Gallic acid were prepared. Aliquot (1 ml) of these were mixed with Folin-Ciocalteu reagent (5 ml) and solution of Na2CO3 (4 ml, 20%). The blue colored was developed and incubate for one hour. Then, absorbance was noted with spectrophotometer at 765 nm. A blank solution (without extract), shoots of intact plant and in vitro micropropagated shoots were also run in similar way. Total amount of polyphenolic were calculated by the following formula and results were expressed as mg of gallic acid equivalent per gram of fresh weight of plant material.

\[ T = C \times \frac{V}{M} \]

Where

- \( T \) = Total phenolic contents in mg GAE/g of plant extract.
- \( C \) = Unknown concentration of plant extract calculated from standard curve in mg/ml.
- \( V \) = Volume of extracts taken in ml.
- \( M \) = Weight of plants extracts taken in grams.

Detection of Alkaloids: Alkaloids were detected by using method described by Tamilselvi et al. (2012). The small portion extracts mixed separately with 0.5ml of dilute hydrochloric acid and filtered. The filtrate was divided into two portions and tested with various alkaloidal agents.

i) Mayer’s test: The Mayer’s reagent (a drop or two) was added in the one portion of filtrate along the sides of the test tube. A white creamy precipitate indicated the test as positive.

ii) Dragendorff’s reagent test: A drop or two of Dragendorff’s reagent was added to the above filtrate by the sides of the test tube. An Orange brown precipitate indicated the test as positive.

2.3 Total flavonide contents total flavonide contents

Plant extracts (1.5 ml) was mixed with 2% ALCL3 (2%) and shake vigorously. After ten minutes the absorbance was noted at 367 nm. Total flavonide contents were determined with quercetin standard curve, and represented as quercetin equivalent (QE) mg/g of fresh weight of plants.

3 Results

The effect of eight different media for micropropagation of cv. PRI-Red and FD 69-1 was compared. The in vitro micropropagated shoots of best micropropagation medium were assessed for the production of polyphenoles. There was a clear correlation between NAA and BAP levels on micropropagation response. Media with BAP lone were showing excellent response for micropropagation and promoted maximum number of shoots per explant with 1.75 or 2.75 mg/l BAP as contained in media MPm6 and MPm7 (Figure 1A). While media with NAA lone showed less or even no shoot induction (Figure 2) as MPm3 medium promoted callus induction rather shoot development. Medium with both BAP and NAA strongly inhibited micropropagation response (Figure 3). Significant variation among media, genotypes and even in their interaction was revealed in analysis of variance table (Table 2).

Among these media, MPm 7 showed an excellent response by promoting maximum number of shoots per explant. Medium, MPm7 gave 7.5 shoots per explant from cv. PRI-Red and 3 shoots per explant from genotype, FD 69-1 (Table 3; Figure 4). This was followed by MPM 6 which gave 5 shoots per explant and 1.8 shoots per explant from PRI-Red and FD 69-1 respectively (Table 3; Figure 4). The remaining media gave comparatively poor response for shoot induction. Minimum number of shoots per explant was observed in PRI-Red on MPm1 and in
FD 69-1 on MPm2 medium. For cv. PRI-Red the response of Mpm4 and MPm5 was statistically non-significant. While media MPm2 and Mpm4 were showing similar micropropagation response for genotype, FD 69-1. Like wise the response to micropropagation of Mpm3 and MPm8 for both genotypes was also non-significant. However the response of MPm2 for PRI-Red and MPm6 for FD 69-1 was similar (Table 3). From this study, it was observed the number of shoots was being increased by increasing the concentration of BAP while lowering the concentration of NAA. However the medium containing same concentration of BAP, and NAA inhibited shoot induction and in this case greenish appearance was observed that could be depicted that might be there is something, which was trying to promote shoot induction while at the same time other thing was inhibiting it (Figure 3). For rooting these in vitro micropropagated shoots were then transferred to root induction medium (Figure 1B). After root induction, plantlets were shifted to Belgium compost for acclimatization and hardening (Figure 1C). In vitro micropropagated shoots of best micropropagation medium (MPm7) and genotype (PRI-Red) whose combination and interaction yielded maximum number of shoots per explant were assessed for secondary metabolites viz., alkaloids, total polyphenolic and flavonide contents in comparison to shoots of intact plant of PRI-Red. Phytochemical analysis of shoots of intact plant and in vitro micropropagated shoots showed that alkaloids were not detected in both in vitro micropropagated shoots and shoots of intact plant while both plants showed positive test for presence of polyphenolics. Shoots of intact plant contained higher amount of total polyphenolics (3.7 GAE mg/g fresh wt.) as compared to in vitro micropropagated shoots (2.8 GAE mg/g fresh wt.). However, slightly higher amount of flavonide were detected in in vitro micropropagated shoots (1.8 mg/g) than shoots of intact plant (1.6 mg/g) (Figure 5).

Table 2 Analysis of variance for in vitro micropropagation of *Solanum tuberosum* explant

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F-value |
|---------------------|--------------------|----------------|--------------|---------|
| Genotype            | 1                  | 44.467         | 44.467       | 225.15**|
| Media               | 7                  | 135.540        | 19.363       | 98.04** |
| Genotype x Media    | 7                  | 29.153         | 4.165        | 21.09** |
| Error               | 32                 | 6.320          | 0.198        |         |
| Total               | 47                 | 215.480        |              |         |

Note: NS = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)

Table 3 Media x genotypes interaction mean±SE: Mean values for shoot induction from explant of *Solanum tuberosum*

| Media | Genotypes | Mean |
|-------|-----------|------|
| PRI-Red | FD 69-1   |      |
| MP1   | 1.500±0.208efg | 0.800±0.115e-h | 1.150±0.189D |
| MP2   | 1.800±0.153de  | 0.200±0.058gh  | 1.000±0.365D |
| MP3   | 0.000±0.000h   | 0.000±0.000h   | 0.000±0.000E |
| MP4   | 3.600±0.404c   | 0.400±0.115fgh | 2.000±0.740C |
| MP5   | 3.900±0.208bc  | 1.600±0.115egf | 2.750±0.525BC|
| MP6   | 5.000±0.493b   | 1.800±0.153de  | 3.400±0.752B |
| MP7   | 7.500±0.611a   | 3.000±0.306ced | 5.250±1.052A |
| MP8   | 0.000±0.000h   | 0.100±0.058h   | 0.050±0.034E |
| Mean  | 2.913±0.515A   | 0.988±0.211B   |      |

Note: Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Lowercase letters represent comparison among interaction means and uppercase letters were used for overall mean.
Figure 1 *In vitro* micropropagation in *Solanum tuberosum* (A) Responses of different micropropagation media (MPm) (B) Rooted shoots of potato (*Solanum tuberosum*) (C) Acclimatized plantlets of potato (*Solanum tuberosum*)
Figure 2 Response of Medium, Mpm3
Note: This medium induced callus induction rather than shoots development.

Figure 3 Response of Medium, Mpm8
Note: This medium showed inhibited shoot induction and a greenish appearance was observed.

Figure 4 In vitro micropropagation response of potato genotypes on eight different Micropropagation media (MPm)
Note: Genotype PRI-Red gave maximum number of shoots per explant on Mpm7 (2.75 mg/l), the most efficient medium for micropropagation. This same medium showed best response to in vitro micropropagation of genotype FD 69-1.
4 Discussion

Potato (Solanum tuberosum L.) is an economically important vegetable crop and is consumed by more than one billion people around the globe (Venkatasalam et al., 2013). Therefore, for the production of disease free seedlings of potato an expedite, efficient and genotype independent micropropagation system is required. In this study, different micropropagation media with varying level and combination of NAA and BAP were studied. Hence a micropropagation medium, MPm7 was selected as best medium for in vitro micropropagation because it gave genotype independent response as well as excellent, expedite and proficient response for both genotypes under study viz., PRI-Red and FD 69-1. Plant growth hormones are the most important factors for regulating shoot development and multiplication. Thus, the inclusion of auxins and cytokinins in the micropropagation medium greatly influences the shoot formation and development. The response and the quality of in vitro cultures are highly influenced by different concentrations and the type of nutrients used in the culture media (Niedz and Evens, 2007). The effect of different nutritive and hormonal constituents of tissue culture media on micropropagation was reported by many researchers (Thirunavoukkarasu and Debata, 2002). Among cytokinins, BAP is proved to be the best responsive for shoot induction (Badoni and Chauhan, 2009; Rahman et al., 2010). Different scientist observed that BAP produces more shoots as compared to kinetin and IAA in micropropagation systems (Carelli and Echaverigary, 2002; Moeinil et al., 2011). However, in this study medium containing BAP lone at concentration of 2.75 mg/l also showed best response to micropropagation and maximum numbers of shoots were achieved in both genotypes of potato from this medium. However, in vitro shoots achieved from medium that was proved to be best, were assessed phytochemically and results of this study showed that total polyphenolic contents were higher in shoots of intact plant than in vitro micropropagated shoots. Alkaloids were not detected in both samples of plant. The variation in the content of the phytochemicals might be due to the different growth conditions and phases of plant development (Nickolova et al., 2013). High contents of polyphenols in shoots of intact plants may be due to increased synthesis of phenols in natural conditions as a defensive reaction to environmental stress (Ramesh et al., 2009). In some other studies also reported that field grown plant leaves contained higher total phenolic than in vitro cultured plants (Hegazai 2011; Sagwan et al., 2011; Singh et al., 2011; Seth and Sarin 2012). Nikolova et al. (2013) also studied that the total content of phenols and flavonoids in the extracts of in vitro cultures were lower than in the extracts of ex vitro and in vivo grown plants.

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