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EXPLOITATION OF VARYING LIGHT REGIMES AND HIGH SUCROSE CONCENTRATIONS FOR *IN VITRO* TUBERIZATION IN POTATO: A STEP TOWARDS DISEASE FREE SEED PRODUCTION

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A B S T R A C T

Potato is the 4th major vegetable crop in our country but more than 90% of the seed is imported which costs heavy economic burden on the foreign exchange. Further, imported germplasm is not well adapted to local climatic conditions and is vulnerable to different types of diseases. *In vitro* manipulations have proved their worth not only for the mass multiplication but also for the production of disease free seed of various crop plants. A proficient *in vitro* micropropagation and tuberization system was established in elite potato cultivars Kuroda and Desiree. In addition to growth hormones, sucrose concentration and light regime appeared to have dominant effect on microtuberization. Maximum number of microtubers was observed in genotype Desiree (2.20±0.20) followed by Kuroda (2.0±0.316) on MS medium augmented with 8% sucrose. Average fresh weight was maximum (0.70±0.063 g) on MS medium augmented with 10% sucrose in genotype Desiree. Likewise, best microtuberization was observed in initial 20 days light (16 hrs light with 8 hrs dark) incubation followed by complete dark incubation. The resultant microtubers were multiplied in pots for the production of minitubers and were tested for disease infestation by ELISA (Enzyme Linked Immunosorbant Assay). The antigen-antibody interaction was detected to be similar to the negative control (0.35-0.528) indicating absence of the viral pathogens. Hence, the developed protocol can be employed for the mass multiplication of disease free seed of elite potato cultivars resulting in reduced dependence on imported seed.

Keywords: *In vitro*; microtuberization; carbon source, disease free; DAS-ELISA.

INTRODUCTION

Potato is a valuable herbaceous plant which is vegetatively propagated from tubers. Pakistan is 18th largest grower of potato but per hectare yield is lower as compared to the developed countries. Soft tissues of the plant attract a wide range of pathogens which may account for 30-75% drop off in the yield (Ahmed *et al*., 2015). Disease incidence due to late blight in major potato growing areas of Pakistan is 24.4- 39.3% (Raza *et al*., 2019). In Pakistan, 83% of the crop losses are due to viral diseases (Hameed *et al*., 2014). Unavailability of suitable climatic conditions for flowering and/or breeding is another limiting agent due to self-incompatibility, tetraploidy and high level of heterozygosity. As a result, we are dependent on imported seed and contribution of locally produced seed is almost negligible. Approximately 13,841 tons potato seed was imported during the last year (GOP, 2021). Repeated sowing of the same seed is not desirable owing to its vulnerability to infectious diseases including common scab, soft rot, brown rot, early blight, stem rot, powdery scab, wilt, black scurf, blackleg, ring rot, bacterial wilt, PLRV, PVY, PVX, PVS and PSA (Spooner., 2013). So, continuous supply of disease free tuber seed is the only desirable remedy to attain sustainable yield of quality crop.

In vitro tuberization is an effective strategy to produce disease free seed potato particularly in warm humid climatic regions where disease dissemination and its spread is beyond control. Various research groups have

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worked it out (Ebadi and Iranbakhsh, 2011; Hossain *et al*., 2015; Smanat *et al*., 2018; Naqvi *et al*., 2019; Mamiya *et al*., 2020) but no considerable breakthrough has been achieved yet. Different growth regulators, osmoregulators and cultural conditions were tested. Aslam *et al*., (2011) reported that 6% sucrose was better for tuberization. Fatima *et al*., (2005) suggested that nodal explant responds better on 6% sucrose under light conditions. Imani *et al*., (2010) also observed that MS medium supplemented with 6% sucrose was best for maximum no. of microtubers (4.20). Hossain *et al*., (2015) standardized that 9% sucrose with 5 mg/L BAP is the best combination for microtuber induction. Smanat *et al*., (2018) observed that jasmonic acid plays important role in tuberization. Naqvi *et al*., (2019) observed that MS medium augmented with sucrose is best responsive to microtuberization as compared with MS medium with growth regulators (BAP and NAA). Mamiya *et al*., (2020) suggested that liquid medium with 10 g/L sucrose, calcium, phosphate, nitrogen in plastic bags was the best for microtuber induction and growth.

Hence, potato seed produced through *in vitro* tuberization will not only be disease free but will also be cheaper having potential to adapt the local climatic conditions. The clonally propagated seed will also provide true-to-type plants and will be genetically similar to the parent genotypes. Further, they have same morphological features and biochemical processes thus no compromise on the desired traits (Fufa and Diro, 2014; Hussain *et al*., 2017). In India, this is a commom slogan that second green revolution in agriculture is linked with microtuber production (Badoni *et al*., 2010). In our country, more than 90% of the seed is imported which is a continuous burden on the National economy. *In vitro* tuberization may prove a great helping hand in this context by contributing seed potato from the local resources. The developed protocol can thus be of pivotal importance for the production of seed potato under local climatic conditions.

MATERIALS AND METHODS

Two potato varieties (Kuroda and Desiree) were kindly provided by Potato Research Institute, Sahiwal and Plant Pathology Research Institute, AARI, Faisalabad and were used in these studies.

Explant preparation: Mature potato tubers of the aforementioned genotypes were incubated at 24°C for sprouting upto 30 days. The tubers with sprouts were sown in peat moss and were incubated at 24°C with 16 hrs light and 8 hours dark regime.

Establishment of explant under *in vitro* conditions: Potato sprouts were surface sterilized and were cultured in the test tubes. For this, sprouts were rinsed with double distilled autoclaved water. Then, dipped in 70% ethanol for 90 seconds and rinsed 3 times with autoclaved distilled water. They were then soaked in 10% commercial bleach with 2 drops of Tween-20 for 5 minutes followed by rinsing with autoclaved distilled water thrice. The sterilized explants were cultured on medium supplemented with 30 g/L sucrose in test tubes.

***In vitro* microtuberization:** *In vitro* growing plants were sacrificed and were cultured in the test tubes having MS medium with concentrations of sucrose 0, 2, 4, 6, 8, 10 and 12%. The pH of the medium was adjusted to 5.70. These test tubes were incubated at 24±1°C under three types of culture conditions A) Complete dark B) 16 hrs. light: 8 hrs. dark. C) 1st 20 days light (16 hrs light with 8 hrs dark) followed by complete dark conditions. These were harvested after 80 days when grown up to proper tubers and data were recorded for number of microtubers and microtuber weight.

***In vivo* minituberization:** Eighty days old microtubers were cultured in the pots containing soil with compost and were incubated at 20°C under light conditions (16 hrs light with 8 hrs dark). Microtubers germinated and were grown up into healthy plants. After 90 days of culture, they were harvested and data were recorded for number of microtubers and their average weight.

Testing for disease infestation: Leaf samples were taken from the *in vivo* growing plants in the pots. Samples were taken from the two positions i.e. top and base of the plant. DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) test was performed using microtiter plate (Clerk and Adams, 1977) following the manufacturer's instructions (Bioreba AG, Switzerland). Polyclonal antibody IgG was diluted in carbonate buffer 1000 times of which 200 µl was used for the coating of wells. Microtiter plate was incubated 4°C for 24 hrs. After this, washing was done thrice with wash buffer at 5 minutes interval and dried on paper towel. The extraction buffer was diluted and used to ground the leaf samples. The plate was coated with leaf sap 200 µl/well and incubated at 4°C for 24 h. Following, three washes with wash buffer. 200 µl of alkaline phosphate conjugated antibody (polyclonal virus specific antibody) was coated in each well and was incubated at 4°C for 24 hrs. Thereafter washing was done with wash buffer for 5

minutes and dried on paper towel. Then, pNPP (p-nitrophenyl phosphate) tablet was dissolved in substrate buffer to make it 1 mg/mL concentration. Plate wells were filled with the substrate buffer and were incubated at 25 °C for 30 minutes. Then, plate was placed in the ELISA reader (EL 800, Biotech. Instrument, Inc., USA) to determine absorbance at 405 nm. Positive and negative controls were used to determine pathogen load.

DATA ANALYSIS

Completely Randomized Design (CRD), in factorial arrangement was used for data analysis. The experiment was performed in five replications per treatment. Analyses of variance were evaluated. Tukey's test was used to analyze difference among means. Interaction effect was also evaluated. IBM SPSS software was used to analyze the data.

RESULTS

Establishment of explant: The potato tubers were incubated at 24±1°C in complete dark conditions. After 30 days of dark incubation the resultant sprouts were sown in peat moss filled pots and were incubated in light dark regime (16 hrs light with 8 hrs dark). *In vivo* as well as *in vitro* growing shoots were used to attain explant tissues for further culture establishment. The apical meristems as well as nodes were cultured on MS medium containing 30g/L sucrose. The test tubes were incubated at 24±1°C under light conditions (16 hrs light with 8 hrs dark). Thirty days old plants were used for further experimentation (Figure 1).

Figure 1. *In vitro* potato plants from sprouts and nodes in MS media with 30 g/L sucrose.

***In vitro* tuberization:** *In vitro* grown plants were shifted to the MS medium with different concentrations of sucrose (0, 2, 4, 6, 8, 10 and 12%) sucrose concentration and were incubated in different light regimes (Detail is given in the Materials and Methods section). The data were recorded and evaluated statistically. Analysis of variance (ANOVA) for number of microtubers and microtuber weight is shown in table 1. The conditions, media and their interactions (C x M) were statistically significant. The genotypes, genotype and media (V x M) and genotype, media and condition (C x V x M) were non-significant. The condition and genotype interaction (C x V) was non-significant for number of tubers and statistically significant for average weight of microtubers (Table 1).

Table 1. Analysis of variance showing effect of media, genotype and culture conditions on microtuberization

Source of variation	Degrees of freedom	Mean squares	
		No. of microtubers per explant	Average weight of microtuber
Condition (C)	2	3.4714**	0.36305**
Variety (V)	1	0.0048 NS	0.06822 NS
Media (M)	6	10.0984**	0.34685**
C x V	2	0.0048 NS	0.10827*
C x M	12	0.5937**	0.06698**
V x M	6	0.0381 NS	0.03423 NS
C x V x M	12	0.0714 NS	0.02475 NS
Error	168	0.2143	0.02403

Mean	209

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

Microtubers started to appear on the aerial shoots, which gradually increased in size (Figure 2). The tuber color was creamy white in dark conditions but greenish in light conditions that turned out to be brownish as grew up. The response of genotypes Kuroda and Desiree was statistically non-significant. For condition (1st 20 days light followed by dark), maximum no. of microtubers were observed in genotype Desiree (2.20±0.20) followed by Kuroda (2.0±0.316) at 8% sucrose. Similarly, microtuber fresh weight was maximum in genotype Desiree (0.70±0.063 g) at 10% sucrose in light conditions (Figure 3 & 4). Microtubers were cultured in pots for the production of minitubers.

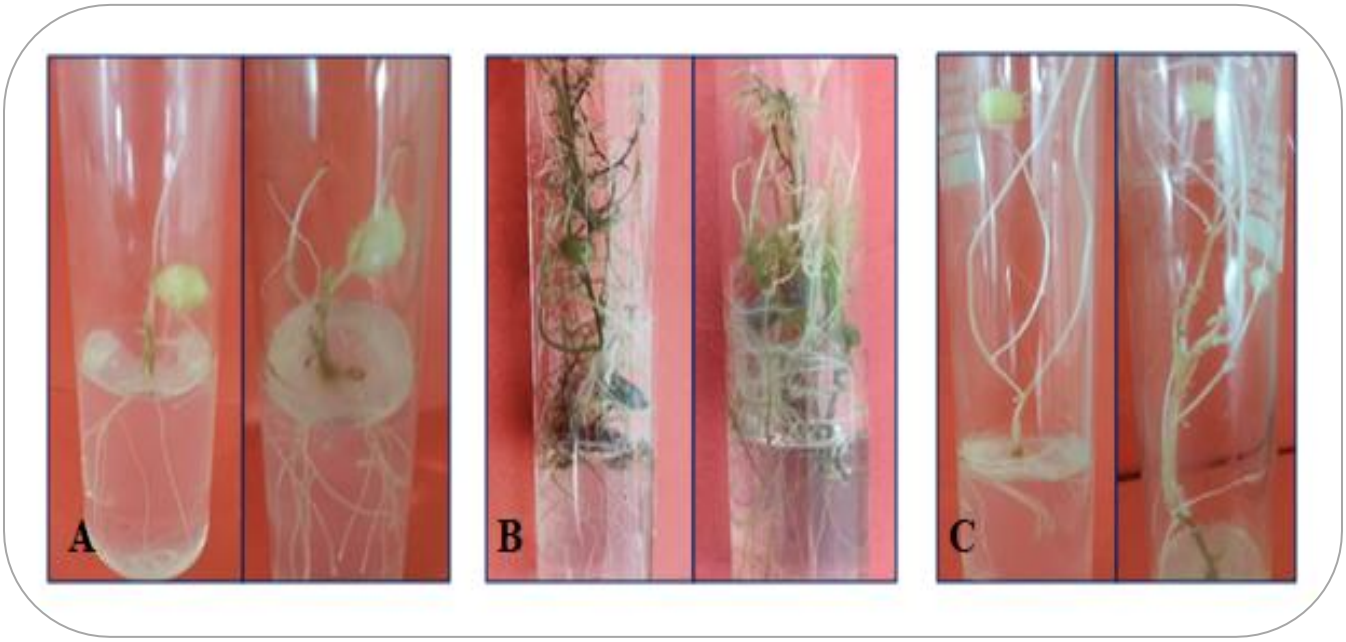

Figure 2. Microtuber induction from nodal explant. A) Microtubers under dark conditions B) Microtubers under light conditions C) Microtubers under initial light incubation (16 hrs light with 8 hrs dark) followed by complete dark conditions

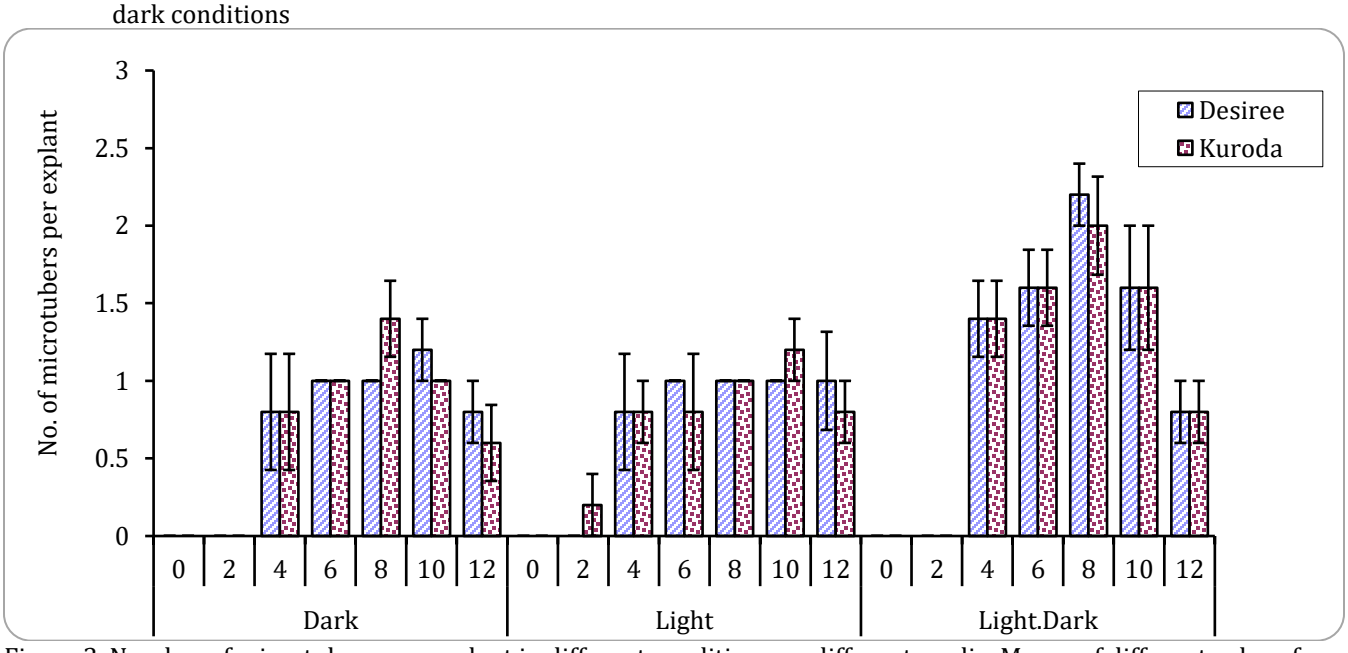

Figure 3. Number of microtubers per explant in different conditions on different media. Means of different values from three replications (n=5) were statistically different using Tukey's test at 0.01 level of significance.

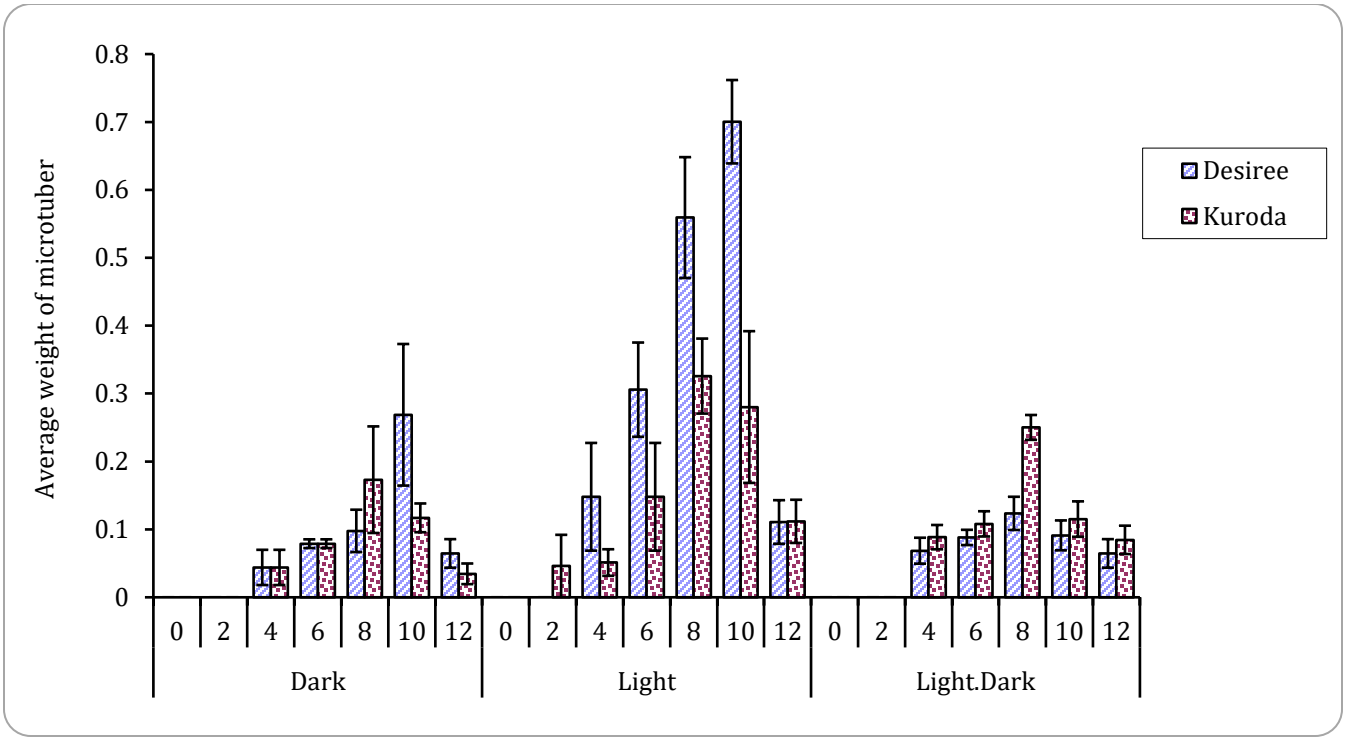

Figure 4. Average weight of microtubers in different conditions on different media. Means of different values from three replications (n=5) were statistically different using Tukey's test at 0.01 level of significance.

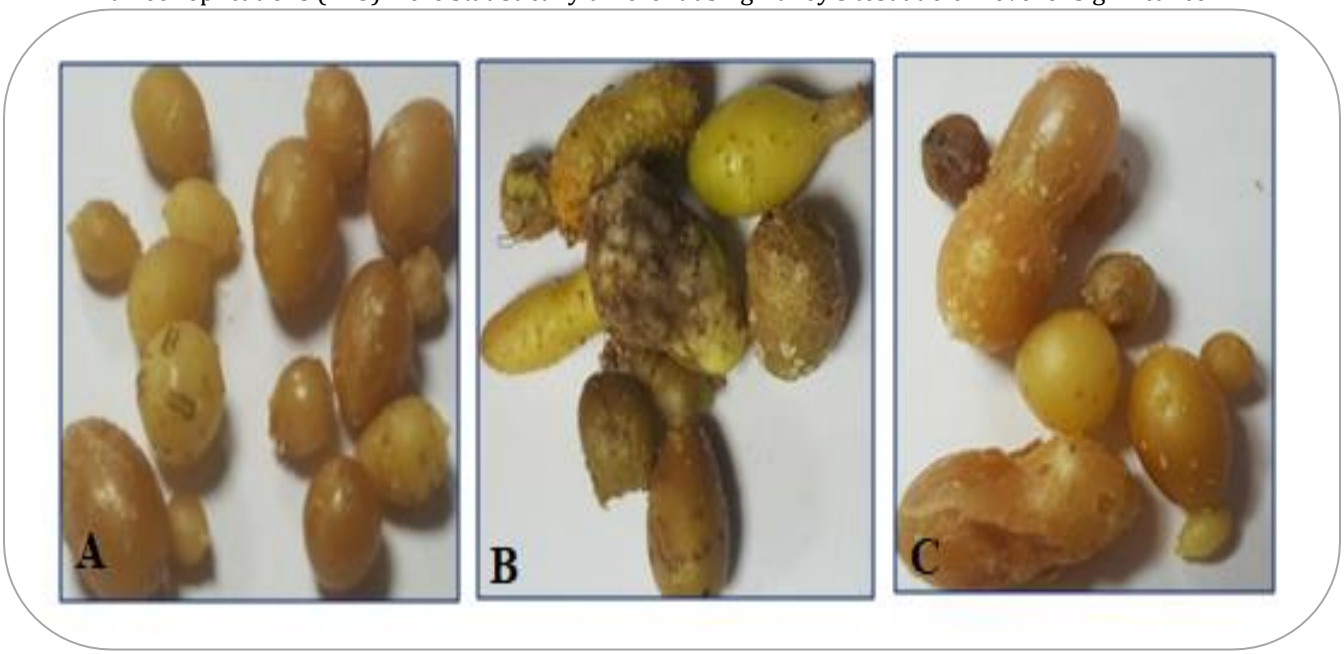

Figure 5. Harvested *in vitro* grown tubers. A) Microtubers under complete dark conditions B) Microtubers under light condition (16 hrs light with 8 hrs dark) C) Micrtubers under light incubation followed by complete dark condition

Minituberization from the microtubers: The harvested microtubers were sown in the pots containing soil with compost and were incubated at 20°C under light conditions (16 hrs light and 8 hrs dark). Microtubers sprouted into shoots which further grew up into healthier plants (Figure 7). After 90 days of culture in the pots, minitubers were harvested. Data were recorded for the number of minitubers and their average weight (g). Number of minitubers varied in different plants. Maximum number of minitubers were observed in genotype Kuroda followed by Desiree (Figure 6).

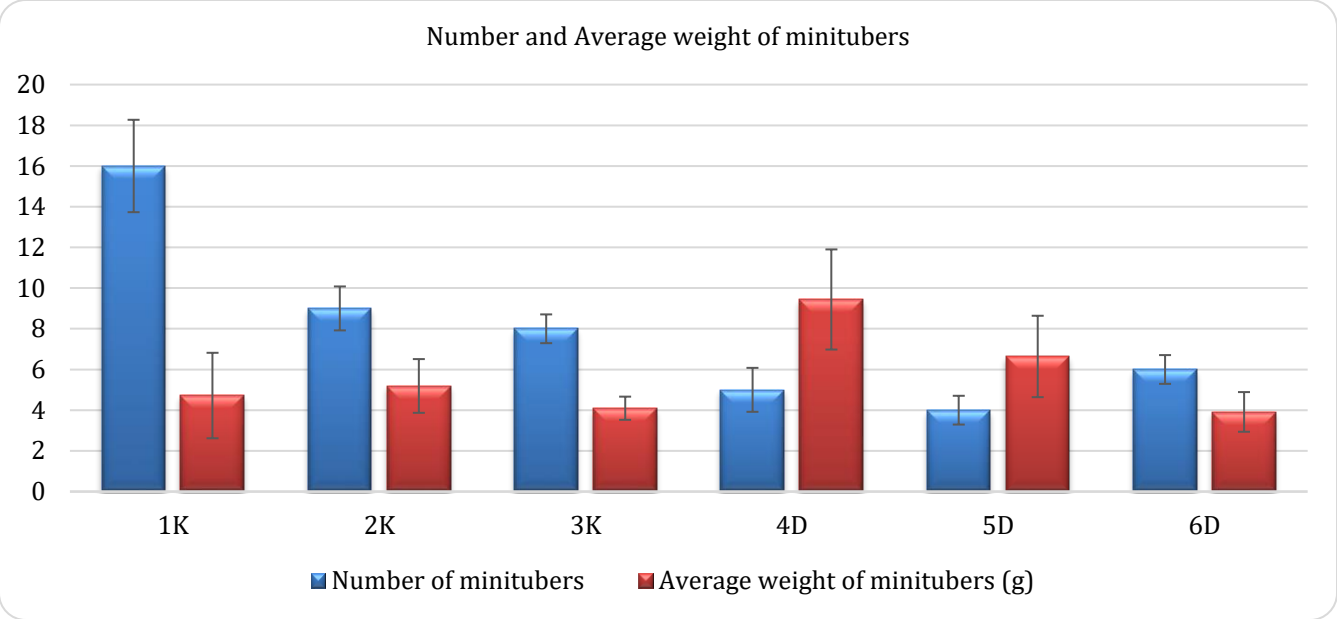

Figure 6. Number and average weight of minitubers in Kuroda and Desiree genotypes. 'K' shows Kuroda and 'D' shows Desiree genotypes.

Figure 7. Plants developed from microtubers were cultured in pots for minituberization and were tested for pathogen infestation through DAS-ELISA

Plants raised from the microtubers were tested through DAS-ELISA for disease infestation. Leaf samples were taken from the nine plants growing in the pots. In addition to plant samples, negative and positive controls were also used. All of the plant samples showed absence of the target protein showing absence of the viral pathogen as no color development and absorbance was determined in the plant leaf samples (Figure 7). OD values of plant samples were similar to the negative control (OD $_{405nm}$ 0.35-0.528) which confirmed that all of the plants raised through this protocol are pathogen free (Figure 8).

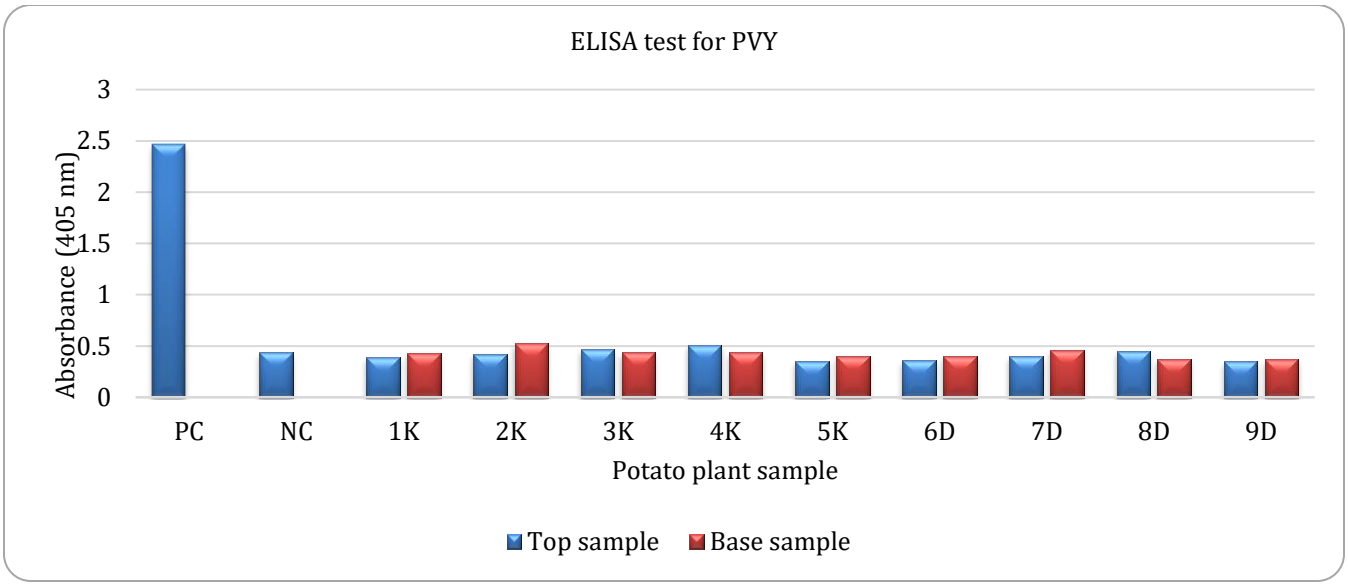

Figure 8. DAS-ELISA test for the Detection of PVY in *in vitro* raised Kuroda and Desiree plants. PC represents positive control whereas NC represents negative control. 'K' represents absorbance value for Kuroda genotype samples whereas 'D' represents absorbance value for Desiree plants.

DISCUSSION

Microtuberization is very important in potato as they are used as seed for its propagation. In Pakistan, disease free high quality microtubers can be grown throughout the year. It is an ideal material for the propagation of plants due to its small size, less weight, easy to transport and high-quality seed potatoes. Different research groups have published, yet our seed contribution of local seed production is negligible. Therefore, more proficient protocols need to be devised. Sucrose is the most cheapest, superior and important determinant of tuberization (Hussain *et al*., 2006). Different sucrose concentrations were studied for two potato cultivars Kuroda and Desiree. Eight percent sucrose concentration was much better for number of microtubers per explant and 10% sucrose concentration displayed the maximum microtuber weight. 2% sucrose did not produce any tuber. The present results are in agreement with (Kanwal *et al*., 2006). They found that MS medium supplemented with 8% sucrose produces 5-6 microtubers per culture having fresh weight of 0.115 g through multinodal explant. 96.6 % cultures showed microtuberization. MS medium supplemented with 3% and 4% sucrose did not produce microtubers. Donbranszki and Mandis, (1993) also found that 8% sucrose was effective for tuberization. Hussain *et al*. (2006) observed 15.6 microtubers per flask under 1st 15 days in light and then shifted to complete dark condition when 90 g/L sucrose was used. 3% sucrose did not produce tubers and explant continued only vegetative growth. One of the critical factor for enhanced microtuberization in complete dark conditions is high production of tuberonic acid. Another critical factor is GA_3 which is reported to be produced during light incubation and it suppresses tuber induction (Jackson, 1999; Alisdair and Willmitzer, 2001). Microtuberization efficiency has been increased by short day or continuous exposure to darkness during culture condition (Dobranszki, 2001; Donnelly *et al*., 2003 and Gami *et al*., 2013). Naqvi *et al*., 2019 found that the MS media without growth regulators had more number of microtubers under both conditions light and dark as compared to the MS media with growth regulators. Hussain *et al*., 2006 also found that no significant effect on microtuber number and size was observed due to BAP. Microtuber induction is mainly effect by sucrose concentration, temperature, light intensity, photoperiod, genotype and inorganic nitrogen (Samant *et al*., 2018; Kumaly *et al*., 2014). If these parameters are changed then number of microtubers, time taken to develop and their quality is affected.

Tuber induction was better if explant was cultured under light conditions for the first 20 days (16 hrs light: 8 hrs dark) followed by complete dark conditions. The initial incubation under light conditions actually supported vegetative growth resulting the establishment of healthy plants. So, light incubation (16 hrs light with 8 hrs dark) followed by dark (complete dark) appeared to

be more appropriate for micro-tuberization than mere light or dark conditions. In light, the physiological processes continue whereas foliar growth is suppressed under dark conditions. Therefore, dark incubation supports tuber induction in the absence or suppressed foliar growth. Hossain *et al*., (2017) also reported that tuberization is attained at 8% sucrose in complete dark incubation at 20°C. They observed that number of microtubers was 1.40 with tuber weight of 370.61 mg. Hoque, (2010) reported that simple MS medium does not respond to tuberization under *in vitro* conditions. They evaluated that MS medium supplemented with 6% sucrose and 4 mg/L kinetin responds better to *in vitro* tuberization. Dark condition took minimum time and showed better response to microtuberization as compared with light conditions. Khuri and Moorby, (1995) proposed that higher concentration of sucrose supports tuber induction and multiplication because a) it is a good carbon source which is easily converted to starch for microtuber growth b) it provides an uninterrupted supply of starch with high osmotic potential. Wang and Hu, 1985; Hussain *et al*., 2006; Aslam *et al*., 2011; Hossain *et al*., 2017 also published similar findings. Fufa and Diro, (2014) reported that MS medium supplemented with 60 g/L sucrose resulted in 2.95 microtubers having 0.06g average weight in genotype Hunde whereas 4% sucrose concentration did not respond at all. Microtuber induction was highly dependent on genotype and sucrose interaction.

Overall 8% sucrose level was found much better for Desiree and Kuroda. Significantly higher number of microtubers was induced at 8% sucrose concentration in MS medium while further increase in sucrose concentration decreased tuber induction and growth. This may be owing to the unfavorable osmotic conditions for water uptake affecting cell turgidity resulting in reduced microtuber formation and growth (Fufa and Diro, 2014). The microtubers obtained are further used for further multiplication in the form of minituber in a green house. Pathogen infestation was tested by ELISA (Enzyme Linked Immunosorbant Assay). ELISA is the most reliable test for the detection of viral pathogens in potato and has been used in numerous research labs for the detection of pathogen (Batool *et al*., 2011; Samsatly *et al*., 2014, Ammar *et al*., 2013 and Iftikhar *et al*., 2021). The OD (Optical Density) determined in the plant samples was close the negative control indicating absence of the viral pathogens in plants raised from micro tubers. Hence, we were able to produce disease free seed potato. The reported protocol can be used for the economical production of disease free seed of different genotypes, well adapted to local climatic conditions thus will prove a step forward for increased production of potato and will be of great help to minimize potato seed import.

CONCLUSION

A protocol for microtubers induction and multiplication was established in elite potato genotypes i.e., Kuroda and Desiree. Microtubers were induced in the MS medium containing 8% sucrose. The resultant micro tubers were successfully grown in pots for the production of minitubers and were tested for disease infestation by DAS-ELISA. No pathogen was detected which confirmed production of disease free seed. The retrieved results are of great value for producing disease free pre basic seed tubers thus will potentially contribute to increased production and decreased potato seed import.

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Contribution of Authors:

Saher Ashraf	:	Conduct research trial and manuscript write up.
Ghulam Mustafa	:	Conceive idea and supervised research.
Muhammad S. Khan	:	Critically reviewed manuscript and provide technical assistance.
Muhammad Aslam	:	Member of supervisory committee