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DEVELOPMENT OF EFFICIENT AND OPTIMIZED PROTOCOL FOR RAPID MICROPROPAGATION OF *Physalis ixocarpa,* A MEDICINAL HERB

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ABSTRACT

The current study describes optimized protocol for in vitro micropropagation of medicinally important accession of Physalis ixocarpa. Plant extract from P. ixocarpa is used for stomach disorder, for purifying blood, as an antidote against local poison and in folk medicine to treat sore throat. Shoots were induced from nodal shoot explants using Murashige and Skoog (MS) media supplemented with different concentrations of benzyalminopurine (BAP). The effect of different concentrations of benzyalminopurine on shoot length, shoot number and per cent shoot induction of explant was observed. Sprouting of shoot explants started within 7-8 days of inoculation. Application of benzylaminopurine @1.5 mg L^{-1} induced maximum shoot length (3.45 cm) with an average of 3.5 shoots explant^{-1} and 96% shoot induction. The healthy cultured shoots were transferred into new culture tubes having rooting media with different concentrations of indole-3-butyric acid (0.25 mg L^{-1}, 0.5 mg L^{-1}, 1 mg L^{-1}IBA). Indole-3-butyric acid at the rate of 1 mg L^{-1} was best for both primary and secondary roots production. In vitro grown plantlets having strong roots were successfully transferred to soil through successive acclimatization which yielded 90% survival rate.

Key words: BAP, IBA, mercuric chloride, micropropagation, *Physalis*.

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INTRODUCTION

Physalis ixocarpa which is commonly known as Tomatillo is belonging to genus *Physalis* and family Solanaceae. The genus *Physalis* has been established by Linneaus in 1753 and it comprises of 100 annual and perennial herbal species (Willis, 1966). According to Sullivan (1984), the unique identification features of this genus are the pendant flowers and an inflated fruiting calyx. Elongated rhizomes and broadly ovate linear leaves are also found in some members of this

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genus. Out of these species only four are cultivated in different parts of the world for their fruit: *P. peruviana* L. and *P. pruinosa* L. are used as jam fruits; *P. alkekengi* L. is used as an ornamental; and *P. ixocarpa* Brot. is used as a vegetable or for sauces.

Endemic weed species of genus *Physalis* are grown in different places in America. Gentry and D'Arcy (1986) enlisted the most important six *Physalis* species growing in the phytogeographic region of Mesoamerica as *P. cordata* Mill., *P. angulata* L., *P. ignota* Britt., *P. gracilis* Miers, *P. pubescens* L. and *P. lagascae* R.S. Most species of the genus are diploid with basic chromosome number = 12 except *P. peruviana* which is a tetraploid (Menzel, 1951). The plant prefers well drained and sandy soil. About 17 accessions of *P. ixocarpa* have been reported from different regions of the world that include PI 309812 (India), PI 360740 (Mexico), PI 662844 (Mexico), PI 512009 (Argentina), PI 512008 (Mexico), PI 512010 (Mexico), PI 512007 (Mexico), PI 5129096 (Mexico), PI 662843 (Mexico), PI 512011 (Mexico), PI 512006 (Mexico), PI 2704599 (Mexico), PI 512005 (Ecuador), PI 662845 (Mexico), PI 662847 (California, United States), PI 662846 (Mexico), PI 291560 (California, United States) (National Plant Germ plasm System USDA). New accession of *P. ixocarpa* with voucher number WD1 was recently reported from District Shangla Khyber Pakhtunkhwa Pakistan by Khan (2015).

The cultivation forms of *Physalis ixocarpa* make Mexico an important center for its diversity as it is grown both as wild and cultivated plant in different parts of the country (Godina *et al.,* 2013). Fruits of *P. ixocarpa* are commonly green in color and consumed in different parts of Mexico, USA and Central America (Mulato-Brito and Pena-Lomeli, 2007). However, purple fruit of *P. ixocarpa* is used in western and central regions of Mexico (Santiaguillo *et al.,* 1998). Importance of tomatillo is also due to the increase in consumption and exports, since this crop is becoming more valuable in other countries. The yield of tomatillo crop in Mexico is low, however the study of autopolyploids in tomatillo offers an opportunity of new breeding strategy for this species (Mendoza *et al.,* 2011). Besides Mexico, other countries are also important for the cultivation of this species including New Zealand, Australia, Africa, Kenya and India (Fisher *et al.,* 1990).

Due to the excessive increase in human population, urbanization, and over exploitation of natural resources, the natural flora in certain areas is rapidly disappearing (Alam and Ali, 2009). Similar situation is faced by *P. ixocarpa* (WD1) which is rare and novel accession of *P. ixocarpa,* limited to mountainous region of District Shangla Pakistan. It is now becoming rare and endangered in Pakistan due to its limited availability and over exploitation (Khan, 2015). Therefore, it is very important to conserve the local flora from being

endangered using proper measures (Ashraf and Akram, 2009; Gilani *et al.,* 2009). Plant tissue culture is one of the most important techniques in biotechnology used for the conservation of endangered and rare economically important plant species. To overcome on biodiversity loss, micro propagation protocol was optimized in this study for mass multiplication and maintenance of biodiversity of this endangered medicinal plant.

MATERIALS AND METHODS

The plants of *P. ixocarpa* (collected from District Shangla KPK, Pakistan) were maintained in pots under greenhouse condition at Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan. These plants served as the explant source for the present in vitro micro propagation experiments. Shoot nodal explants were used as experimental material. Expanding and emerging leaves were removed from the shoot with biracial needle. The shoot was cut into small portions of 2-3 cm long with sterile forceps and carefully transferred to the culture flask. The explants were washed thoroughly in running tap water for 30 minutes and then kept in liquid detergent (Tween 20) for 30 minutes for surface sterilization.

The sample was then washed with running tap water to remove any traces of the detergent. The explants were then dipped in 70% (v/v) ethanol for 1 minute and surface sterilized with 0.1% (w/v) mercuric chloride for 2 minutes followed by five rinses with distilled water in laminar air flow cabinet. The basal medium used for the culture was MS medium with sucrose 3% and 0.8% agar and growth hormones (Murashige and Skoog, 1974). Under sterile condition, the explants of suitable size were inoculated in culture test tubes containing MS medium with different concentrations of BAP (0, 0.5, 1 and 1.5 mg L^{-1}) for shoot proliferation. After inoculation the mouth of test tubes were quick flamed, tightly capped and properly sealed with kiln film to avoid entry of external air. The test tubes were transferred to growth room after proper labeling clearly mentioning media code, date of inoculation.

Data was recorded after 23 days of culture and only shoots greater than 2 cm were considered for recording the data. The explants with newly form shoots were taken out after 24 days of culture period. The newly formed shoots were then transferred to rooting media containing different concentrations of IBA (0.2 mg L^{-1}, 0.5 mg L^{-1} and 1mg L^{-1}). Each treatment was replicated 20 times. Data was noted after 30 days of culture. The cultured tissues were incubated under specific condition i.e. control temperature, light quality, air circulation and humidity for specific time. Temperature of

culture room (Polylux XL, GE Britain, 36W) was 25± 10 °C with16 hrs photoperiod with light intensity of 2000-2500 lux (Provided by Polylux XL, GE Britain, 36W).

Prior to final transfer to the soil after 30 days of culture on rooting media, the plantlets were transferred to plastic pots for their hardening and acclimatization. Plants with newly formed roots were taken out from the test tubes with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in warm water to remove the any traces of solidified agar. After this treatment the plants were carefully planted in plastic pots containing 1:1 mixture of soil and farmyard manure and thoroughly watered it. The plants were kept under poly house for ten days with temperature of 31°C and 80% humidity. During this duration the plants were thoroughly watered with the help of sprinkler to maintain the required level of humidity. The plants were then transferred to shade house with less humidity level and indirect sunlight and watered it two times a day i.e. morning and evening to prevent wilting.

Statistical Analysis

Data was analyzed using statistix 8.1. The one- way analysis was done for statistical comparison, and p values< 0.05 were considered significant.

RESULTS AND DISCUSSION

Analysis of the data indicated that soaking of shoot explants in 0.1% mercuric chloride for 5 minutes resulted in 100% mortality rate. When exposure time was reduced to 3 minutes, the mortality rate decreased to 20% with contamination rate of 12%. Mortality rate of 0% with contamination rate of 14% was observed by reducing the sterilization time of explants to 2 min. After one week of all the explants showed the signs of the shoot proliferation and new buds started to appear from the axial of the leaves and bud developed in to shoot. All treatments produced shoots from explants; however rate of shoot induction differed significantly in various concentrations of BAP. Difference in BAP level produced a significant response upon the length, number of shoots per plant and also on percent shoot induction at $p<0.05$ (Fig. 1). Similar results were also reported by Sudharson *et al.* (2014) and Ismail *et al.* (2012). The data defected in the Table-2 revealed that sprouting of shoot explants started within 7-8 days of inoculation. Application of BAP (0.5 mg L^{-1}) produced an average of 3.75 shoot number per explant having shoot length of 2.5 cm. With increase in the concentration of BAP to 1 mg L^{-1}, the average shoot length reached to 4.2 cm and average shoot number decreased to 2.9 per explant. When the concentration was increased to 1.5 mg L^{-1}, the average shoot number was 3.5 per explant with shoot length of 3.45

cm. In the present study shoot length was directly proportional whereas shoot number was inversely proportional to concentration of BAP. The finding corroborate the result of result of Singh and Arora (1995) who reported increasing the concentration of BAP results in decrease in shoot length and increase in number of shoots per explant.

The hormone free culture media exhibited no significant effect on the shoot proliferation of the explant by producing average shoot number of 1.5 per explant and shoot length of 1.3 cm. Different concentration of BAP produced varying degree of percent shoot induction under in vitro culture conditions. At 23 days after culture, 0.5 mg.L^{-1} revealed 90% shoot induction which increased to 93% and 96% by the application of 1 mg L^{-1} and 96% at 1.5 mg L^{-1} BAP respectively. Afroz *et al. (*2009) reported that maximum number of shoot was observed on the media containing 1 mg L^{-1} BAP while Ramirez and Alejo (1991) stated that highest number of shoot formation was produced on media containing 2.5 mg L^{-1} BAP and 1 mg L^{-1} NAA. Uddin *et al.* (2006) investigated that the highest number of shoots was produced on medium containing 3 mg L^{-1} BAP while Afroz *et al*. (2009) obtained highest shoot regeneration of 95% using BA and coconut water with 32 mean numbers of shoots on MS medium from nodal explants of *P. minima.* Similarly, Sheeba et al. (2010) reported 84% direct shoot regeneration with 19 mean number of shoots on MS medium supplemented with 2.0 µM BA. After 23 days of the explants on shooting media, the healthy cultured shoots were transferred in to new culture tubes containing rooting media. The rooting media was supplemented with different concentration of IBA (0.25 mg L^{-1}, 0.5 mg L^{-1}, and 1 mg L^{-1}. IBA free media was used as Control.

Analysis of the data shown in Table 3 revealed that culture media containing IBA (1mg.L^{-1}) showed better root re-generation as compared to other treatments. The data further indicated that application of the IBA (1mg L^{-1}) induced primary roots of an average 2.3 cm length and secondary roots with an average number of 22.26 per shoot respectively (Fig. 2D). The rooting media containing IBA (0.5 mg L) did not initiate the formation of the secondary roots and produced only primary roots with an average of 1.2 per shoot under invitro culture condition. However the hormone free media and the media containing IBA (0.2 mg L^{-1}) did not induce rooting. Statistical analysis of the data revealed that significant ($p<0.05$) differences were observed in root number, root length and duration of root formation by applying different concentration of IBA to the shoots under control condition.

Application of IBA at 1mg L^{-1} had significant ($p<0.05$) effect on primary and secondary root formation along with their length as compared to other treatments. Our results indicated that maximum

number of roots was observed on the half strength media supplemented with 1 mg L^{-1} IBA within 30 days. Our results are supported by Intzaar *et al.* (2013) who reported that maximum number of roots as well as the long roots was obtained on rooting media supplemented with 0.5 micromole of IBA.

Acclimatization of the tissue culture plants is the most critical step in micro propagation. The plants grown under in vitro condition are very sensitive and difficult to directly face ambient environmental conditions (Bhojwani and Razdan, 1992). The mortality rate of these plants depends on first and second hardening stage. First hardening stage is most critical in which the plants are kept in poly house for 10 days while in the second hardening stage survival rate is better than first as the plants are comparatively hardened during the first hardening stage. In this study, during the first hardening stage, the rooted plantlets were transferred to the pots containing farmyard manure and soil (1:1 ratio) prior to the final transfer to the soil. In both stages of hardening, the transplanted plant showed good survival rate. The first hardening stage showed 79% survival rate while in the second hardening stage 82% of the total plants survived.

Table-1. Effect of different dipping time intervals (0.1% mercuric chloride) on shoot tips responses of *Physalis ixocarpa*

Explant	Percent Concentartion of sterilant	Time exposure	Percent Response		
			Contamination	Mortality	Survived
Shoot Tips	0.1	5	-	100	0
	0.1	3	12	20	80
	0.1	2	14	0	100

Table -2. Effect of different concentrations of BAP on number of shoot per explant and shoot length (± represent standard deviation from mean of the 30 samples)

Growth Regulator	Shoot Number				Shoot length (cm)			% Shoot induction
BAP(mg.L^{-1})	N	Min	Max	Mean ±SD	Min	Max	Mean ±SD	
0.5	30	3.0	5.0	3.75±0.86	1.2	3.3	2.5 ±0.64	90
1.0	30	2.0	4.0	2.90±0.72	2.1	6.6	4.2700±1.58	93
1.5	30	3.0	5.0	3.55±0.61	1.5	6.5	3.4500±1.38	96
Control	30	0	3.0	1.65±0.82	0	2.3	1.3±0.52	30

Table-3. Effect of different concentration of IBA on root length, number of primary and secondary roots (± represent standard deviation from the mean of 20 samples)

IBA mg.L^{-1}	No of Primary Root				Length of Primary Root (cm)			No of Secondary Root			% Root induction	Days for Root Induction
	N	Min	Max	Mean±SD	Min	Max	Mean±SD	Min	Max	Mean±SD		
0.2	20	0	0	0	0	0	0	0	0	0	0	65
0.5	20	0	2	1.2±0.77	1.2	3.3	2.426±0.66	0	0	0	65	32±0.833
1.0	20	1	3	2.33±0.72	1.4	4.5	2.98±0.26	19	25	22.26±1.7	85	29±0.961
0.0	20	0	0	0	0	0	0	0	0	0	0	65

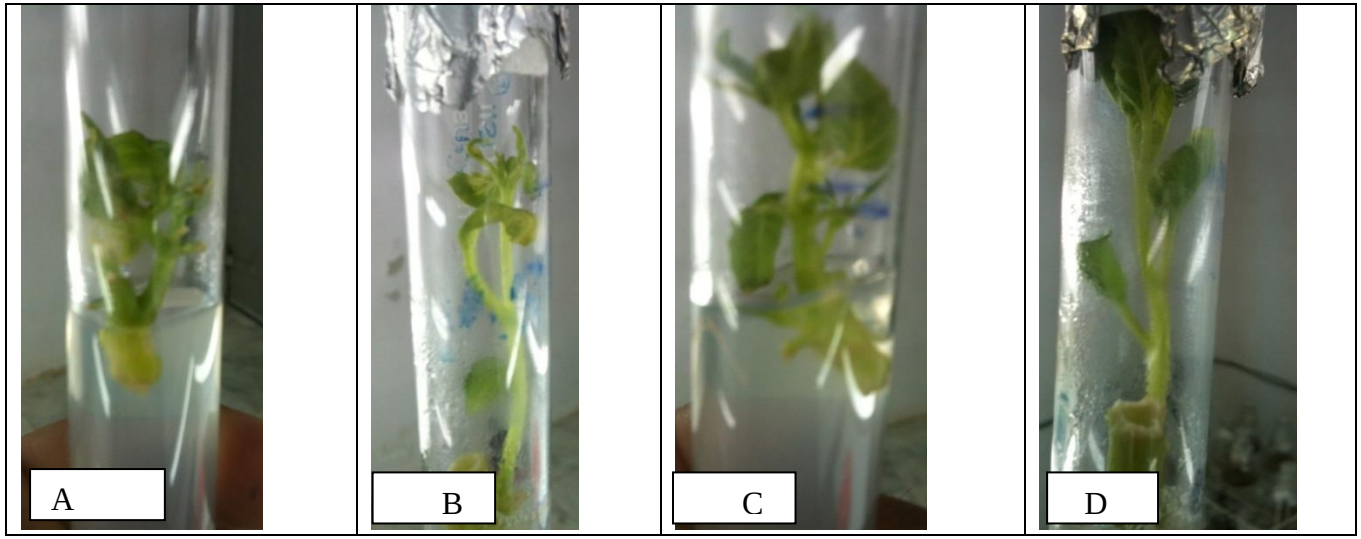

Figure 1. In vitro shoot formation from shoot nodal explants
A = MS media without BAP, B = MS media with 0.5 mg L^{-1} BAP, C = 1 mg L^{-1} BAP, D = 1.5 mg L^{-1} BAP

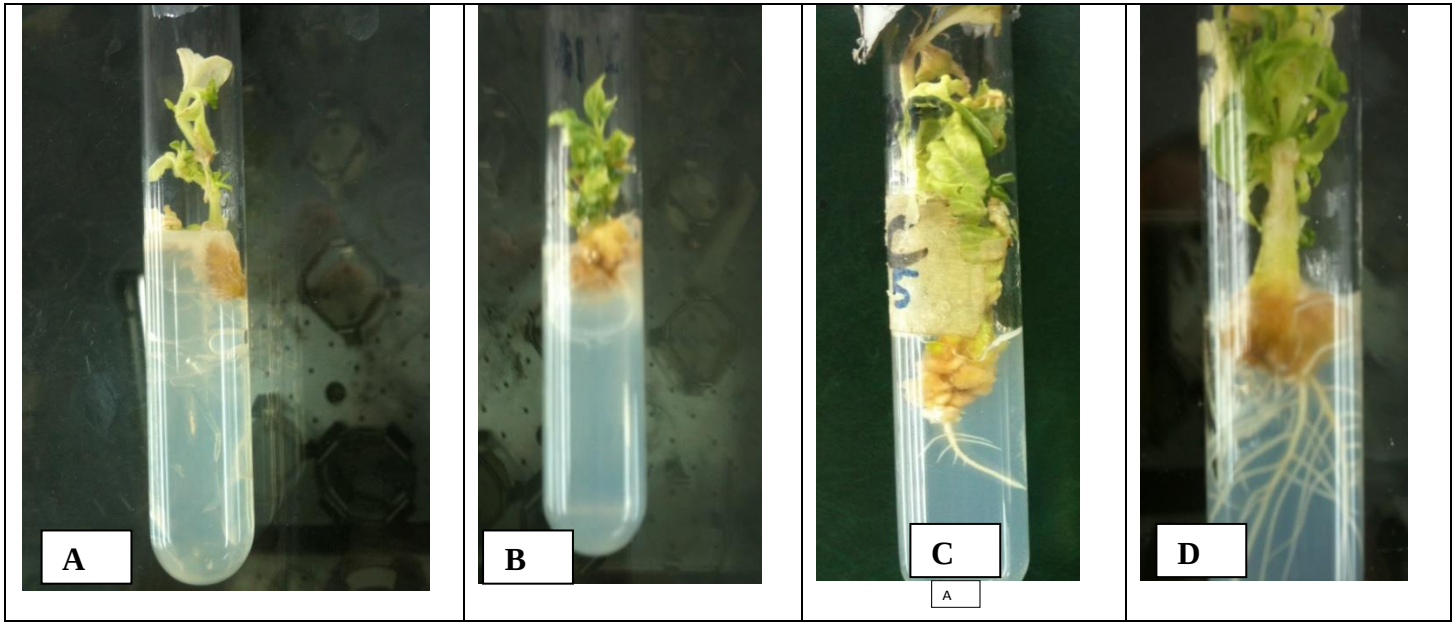

Figure 2. In vitro root formation from shoot nodal explants
A= half MS Medium without IBA, B= half MS Medium with 0.2 mg L^{-1} IBA,
C = half MS Medium with 0.5 mg L^{-1} IBA, D= half MS Medium with 1 mg L^{-1} IBA

CONCLUSION

Physalis ixocarpa is medicinal plant of significant importance. In Pakistan it is found in district Shangla of Khyber Pakhtunkhwa. Due to the excessive increase in human population, urbanization, and over exploitation of natural resources, this accession of *P. ixocarpa* has now become limited and endangered. In this study, we have optimized the simplest and efficient mircro propagation protocol for rapid mass multiplication, and maintenance of biodiversity of this endangered medicinal plant.

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