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Optimization of Explant and Plant Growth Regulators to Increase the *Invitro* Regeneration Frequency of Potato (*Solanum tuberosum* L.) *cv*. SH-5

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Abstract

Efficient *in vitro* culture system was standardized for potato (*Solanum tuberosum*) *cv*. SH-5. Callus formation and plant regeneration were obtained by culture of potato leaf discs and internodal stem segments on MS medium supplemented with different growth regulators. MS medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) at 4 mg L^{-1} showed the best results for callogenesis. There was 96% callus induction at 4 mg L^{-1} of 2,4-D by ,leaf explants as compared to 74% exhibited by intermodal stem segments. Embryogenic calli regenerated shoots on MS supplemented with 2 mg L^{-1} BAP + 2.5 mg L^{-1} NAA, had the highest efficiency (64% and 35%) and highest number of shoots per callus (5 and 2) from leaf and stem explants, respectively. Regenerated shoots were separated and rooted on MS medium. The highest rooting response (96%) was observed in the shoots originating from leaf callus as compared to 70% in shoots derived from stem callus. Rooted shoots were acclimatized in the glasshouse for plantation in the field.

Key words: 2-4,D, Callus, Embryogenic calli, Meristem, Regeneration, Somatic embryogenesis

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Introduction

Tuber is an edible part of potato plant, which is developed from underground stem known as stolon. It is used as an inexpensive food, animal feed, industrial raw material and seed tuber (Khurana & Naik, 2003). It provides more protein and calories than other food crop (Khalafalla et al., 2010). Potato tubers often give high yield acre-1 than any grain crop and are consumed in a number of ways at homes (Elaleem et al., 2009). Potato plays an important contribution in human diet along with wheat and rice. It provides 2-3% protein, 80% water and 18% carbohydrates (Rafique et al., 2004).

Availability of quality seed is major production constraint in potato (Rafique et al., 2004). Potato is propagated largely by tubers where as true seed propagation is carried out for breeding purposes. Potato crop is affected by a large number of abiotic and biotic factors. Pests and diseases are major abiotic factors that affect potato crop. The major diseases include late blight, powdery scab and black scurf (De Jong et al., 2011) that may cause severe economic losses (Li et al., 2018).

In this regard, micropropagation techniques may be effectively used to eliminate the pathogen and allow multiplication of healthy seed which may be used as basic seed for multiplication in field (Afzal et al., 2019). It also reduced 50% of seed cost as compared to imported potato (Karim et al., 2011). Plant tissue culture also offers a proficient methodology for the production and rapid propagation of pathogen-free material and germplasm conservation (Jafari et al., 2019). Transgene introduction also require an optimized method of calli regeneration for successful delivery of gene (Kaur et al., 2018). SH-5 is an important potato variety of Pakistan released in 2006 for commercial cultivation with a high yield potential of 48700 kg ha-1. It is a white skinned variety which is well adapted to local climatic conditions (http://www.agripunjab.gov.pk). The in vitro culture studies for SH-5 were scarce. The tissue culture and development of regeneration protocol through embryogenic calli is a pre-requisite to most of the novel techniques of plant biotechnology. In this connection, the present research is aimed at optimization of an effective in vitro plant regeneration procedure for SH-5. A reproducible plant regeneration protocol established in the present study will help exploit future biotechniques for this variety.

Materials and Methods

The research work was carried out in Somatic Cell Genetics Lab, Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad.

Plant material

Tubers and micropropagated plants of potato *cv*. SH-5 were obtained from Agricultural Biotechnology Research Institute (ABRI), *Ayub Agricultural* Research Institute (AARI), Faisalabad, Pakistan.

The *in vitro* cultures were established and maintained using stem node explants sub-cultured every 3–4 weeks. Nodal segments were dissected and cultured on MS medium in glass jars and test tubes. These cultures were maintained at 20°C ± 2°C in light under a 16 h photoperiod.

Sterile distilled water was used for washing of potato tubers. These tubers were then kept in craft paper bags and stored under dark conditions at 25°C for 3 months for sprouting (Kaur et al., 2018). The sprouted tubers were then transferred to pots (8cm diameter) containing clay and placed under greenhouse conditions to obtain healthy plants. After one month the emerging plants were shifted to larger pots (15cm diameter) with tubers attached in clay-filled pots and maintained in the glasshouse at 25°C ± 2°C with a light intensity of 2500 lux and 16-h photoperiod provided bycool-white fluorescent light. Leaves and internodal stem segments of these axenic plants were used as explant sources throughout the study.

Assessment of media and culture environment

The basal medium used for callus induction, shoot regeneration, root formation and further *in vitro* multiplication was MS (Murashige and Skoog, 1962) medium. Sucrose was added as a source of carbon at the rate of 30 g L^{-1}. Culture medium was adjusted to a pH of 5.8 and solidified with 8 g L^{-1} of agar. Aliquots (10 ml) of each medium were dispensed in to each test tube (18×150 mm) covered with polypropylene plastic sheet. The powder-round, screw capped glass jars of 175 ml capacity were also used accommodating 40 ml of culture medium in each jar. The culture media for all investigations were sterilized by autoclaving at 121°C at 15 psi for 20 minutes.

Axenic shoot cultures

Stem segment explants (1.0 cm lengths) each with a node or with an apical meristem were maintained (four segments per jar) in 40 ml aliquots of MS basal medium contained in 175 ml capacity glass jars. The cultures were kept at 20ºC ± 2ºC in the light under a 16 h photoperiod (cool white fluorescent tubes) for complete plant development.

Explant sterilization and callus initiation

The youngest, fully expanded leaves and stems from one-month old glasshouse-grown plants of SH-5 were used for establishment of callus cultures. These explants were surface sterilized by immersion in 10% (v/v) sodium hypochlorite solution (NaOCl) for 20 minutes, followed by 3-4 rinses with autoclaved reverse-osmosis water. Young leaves were dissected into smaller segments each of 1cm^2 size. Each test tube filled with different media combinations accommodated single explant. Whereas, four leaf segments were cultured in one jar containing 40 ml of culture medium. MS medium was supplemented with different concentrations of 2, 4-D viz 0, 2, 4, 6 and 8 mg L^{-1} for callus induction. Each medium treatment was replicated ten times for each explant. The same growth conditions, as for axenic cultures, were maintained for incubation of cultures.

Maintenance of callus cultures

Leaf and stem-derived callus cultures were transferred to respective fresh media after 28 days of callus initiation. Calli that showed slow growth or browning were discarded. Two to three subcultures were performed before shifting calli to plant regeneration media. All callus parameters including callus induction frequency, callus morphology, callus quantity, callus texture, callus color, callus texture, fresh weight of callus, number of shoots per callus, regeneration frequency and rooting frequency were recorded during this period of callus development and proliferation.

Plant regeneration from callus cultures of cv. SH-5

Callus cultures induced from leaf and stem explants, after 2-3 subcultures on same fresh callus induction media proliferated to be transferred to regeneration media. The calli (4-6 weeks old) were sectioned into small pieces (each weighing 0.5 g of fresh weight approximately); a single callus section was cultured into each culture tube containing 10 ml of regeneration media. In case of glass jars, four callus pieces were cultured in each having 40 ml of regeneration media. Different culture media were assessed for plant regeneration response of leaf and stem-derived calli of *cv*. SH-5. These are MS + 2 mg L^{-1} BAP + 2.5 mg L^{-1} NAA, MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, MS + 0.5 mg L^{-1} BAP + 2 mg L^{-1} NAA, MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} IAA, MS + 4 mg L^{-1} KN + 0.5 mg L^{-1} NAA

Each culture medium treatment was replicated ten times. The same culture incubation conditions were maintained as described in above section. The parameters related to plant regeneration response evaluation were recorded 4 weeks after culture on regeneration media.

Rooting of in vitro-regenerated plants

Regenerants of SH-5 (2-3 cm long) derived from leaf and stem explants were transferred individually to glass jars (screw capped) for root induction. Each glass jar contained 40 ml of MS medium. The plants were kept on this medium for 2-4 weeks till vigorous rooting. The cultures were incubated following the conditions as mentioned in above section. The regenerated rooted plantlets with 4-5 leaves stage were transferred from culture room and kept in room temperature (approx. 30ºC) for 4-5 days. The plantlets were then removed from the culture tubes and all the adhering media were carefully washed out so that, the root damage was the least. Washed plantlets were planted into small plastic pots containing sterile soil, sand and decomposed cow dung at the ratio of 1: 1: 1.

Traits evaluated for callogenesis and plant regeneration.

Callus induction frequency: Assessed as percentage of the total number of explants forming non-necrotic callus.

Callus morphology: Assessed on the basis of callus color, callus quantity and callus texture. Callus quantity: Visual recording of quantity of callus as ++++ (excellent callus induction), +++ (best callus

induction), ++ (good callus quantity), + (low callus formation) and ¬¬¬¬¬¬¬¬¬— (no callus). *Callus color*: Different callus colors viz; yellow, cream, greenish etc were visually

recorded for each explant type cultured on each medium. *Callus texture:* Different callus textures viz; friable, watery, compact, nodular etc were also visually recorded for each explant type cultured on each medium. Fresh weight of callus: Recorded by weighing the callus on electronic balance in laminar flow cabinet.

Number of shoots per callus: Recorded as mean number of shoots produced per individual callus after 4-6 weeks culture on regeneration medium. *Regeneration frequency*: Calculated on the proportion (%) of calli giving one or more shoots. Rooting frequency: Recorded as proportion of shoots (%) cultured on rooting medium which produced roots.

Establishment of regenerated plants in the glasshouse

The regenerants with fully developed roots (derived from leaf and stem calli) approximately 4-5 cm in height were removed from rooting medium (4-6 weeks after culture on rooting medium). The roots were washed thoroughly with tap water to get rid of agar and transferred to pots. The handling measures were as described in above section. These plants were covered with clear plastic bags, the bag corners were cut opened after 5 days and these were removed after 14 days. This was an effective strategy to prevent rapid moisture loss from newly-transferred in vitro regenerants to pots. After one month, the plants were transferred to 15 cm diameter pots for further evaluation of morphological characters.

Statistical analyses

The experiment was conducted in CRD with 10 replications. Each test tube was considered as a single replication. Statistical analysis of growth response data regarding evaluations of media, formation of callus and plant regeneration responses of cv. SH-5 was performed using standard analysis of variance (ANOVA) (Snedecor and Cochran, 1989). The means and standard error of means (SEM) were calculated and statistical significance between the mean values was assessed using LSD test at 5% level of significance (Steel et al., 1997).

Results

Analysis of variance

Analyses of variance showed significant variation ($P \leq 0.01$) for treatments, explants and their interaction (Table 1). Significant interactions showed that explants (disc and intermodal) changed their relative ranking across various treatments. Thus various traits values were compared across the specific plant growth regulator treatment.

Explant response for callogenesis

Callogenes started from the cut edges of the explants. Both the explants leaf and stem segments produced callus on all 2,4-D concentrations ranging from 2–8 mg L-1. In comparison, leaves responded well to callogenes is than intermodal stem segments (Table 2). Leaf explants showed 64.00% callus efficiency as against 46.00% of intermodal stem segments. Callus induction response was the highest (96.00%) at 4 mg L-1 of 2,4-D. Whereas, the lowest call (54.00%) was induced at 8mg L-1 of 2,4-D concentration from leaf explants. Stem explants produced maximum callus (74.00%) at 4mg L-1of 2,4-D and the lowest callus (44.00%) was observed on mg L-12,4-D.

Effect of 2,4-D on callus formation

Callus induction from both the explant sources was evaluated at different levels of 2,4-D. Lower levels of this auxin favoured higher callus production in leaf as well as stem explants. No positive effects were observed on callus initiation and proliferation by using the highest concentration of 2,4-D (8mg L-1) (Table 2). Instead it induced non-embryogenic calli and as a result embryogenesis was hampered. Among the low levels, 4mg L-1, 2,4-D was observed to be the most effective level for callus induction producing 96.00% and 74.00% callus in leaf and stem explants, respectively. Overall comparison of 2,4-D levels for the two explants indicated the callus induction values of 85.00%, 73.00% and 57.005%, when 4, 2 and 6 mg L-1 dose was used, respectively in MS medium.

Table 1. Analyses of variance for callus fresh weight of callus (FWC), percent callus induction %, number of shoots per callus, regeneration frequency % affected by various plant growth regulator treatments and different explants of potato cv. SH-5.

S.O.V	**DF**	**Mean sum of Square**			
		FWC	C%	NS C^{-1}	RF%
Treat (T)	4	4.59*	2.60*	40.802*	6091*
Explant (E)	1	0.09*	3.24*	69.76*	9990*
T × E	4	0.09*	2.59*	4.26*	579*
Error	90	0.00	0.20	0.00	7.00

Where * is highly significant when $P \leq 0.01$

2,4-D	**Percent callus induction**		**Mean**
	Leaf	Internodal segments	
0	0.00f	0.00f	0.00
2	0.86ab	0.6cde	0.73a
4	0.96a	0.74bc	0.85a
6	0.64cd	0.5de	0.57b
8	0.54de	0.44e	0.49b
Mean	0.64a	0.46b	

Table 2. Callus induction percentage of potato *cv*. SH-5 as influenced by various concentrations of 2,4-D

Values are means of 50 observations explant per treatment

Morphological characterization of callus of Potato cv. SH-5 derived from leaf and internodal stem segments Callus induced from leaf and internodal stem segment explants was transferred to the same callus induction media for further growth and proliferation. Wide variations were observed for callus quantity, color and texture on different callus culture media. Leaf and stem segments cultured on MS salts fortified with lower 2,4-D levels (2 and 4 mg L^{-1}) produced light green-yellowish friable-watery calli. The quantity of callus induced at these lower auxin levels was also high (+++) (Table 3).

Fresh callus weight

Fresh weight of callus was scored four weeks after culture on different callus induction media. Both the explants leaf and stem segments produced callus on all 2,4-D concentrations ranging from 2-8 mg L^{-1}. While comparing both the explants, leaves produced more callus weight than internodal stem segments (Table 4).

Leaf explants showed 0.88g callus weight as against 0.82g of intermodal stem segments. The maximum callus weight (1.17g) was produced at 2mg $L^{-1}$of 2,4-D. Whereas, the lowest callus weight (1g) was observed at 8mg $L^{-1}$of 2,4-D concentration from leaf explants. Internodal stem explants produced maximum callus weight (1.14g) at 4mg $L^{-1}$of 2,4-D and the lowest callus weight (0.94g) was observed on 8mg L^{-1} 2,4-D.

Callus formation from both the explants was evaluated at various levels of 2,4-D. The amount of callus produced generally decreased with a gradual increase in the concentration of 2,4-D from 2mg $L^{-1}$to 8mg L^{-1}. Among the low levels, 2mg L^{-1}2,4-D proved the most effective level producing 1.17g callus weight in leaf explants. While 4 mg L^{-1}2,4-D was observed to be the best level producing 1.14g callus weight in stem explants. Overall comparison of 2,4-D levels for the two explants indicated the callus of weight values of 1.14g,1.08g and 1.05g when 4, 2 and 6 mg $L^{-1}$of 2,4-D was used, respectively in MS medium

Table 3. Morphological characterization of callus of Potato *cv*. SH-5 in leaf and internodal stem segments

(MS+ 2,4-D mg L^{-1})	**Callus morphology / Explant**					
	Leaf			Internodal stem segments		
	Callus amount	Callus color	Callus texture	Callus amount	Callus color	Callus texture
0	–	–	–	–	–	–
2	++++	Greenish	Compact	++	Light Green	Friable
4	+++	Light Green	Friable	++++	Yellow	Friable
6	++	Light Yellow	Watery	+++	Yellow	watery
8	++	Light Yellow	Watery	+	Yellow	Watery

(-) No Callus; (+) Poor Callus; (++) Good Callus; (+++) Best Callus; (++++) Excellent Callus
Values are means of 50 observations per explant per treatment

Table 4. Influence of 2,4-D levels on fresh weight of callus (g) of potato *cv*. SH-5

Treatment(MS+2,4-D mg L^{-1})	Fresh Weight of Callus (g)		Treatment Mean Values
	Leaf	Internodal Segments	
0	0.000f	0.000f	0.000f
2	1.17a	0.99de	1.08b
4	1.15 a	1.14ab	1.14a
6	1.08bc	1.02cd	1.05b
8	1.00de	0.94e	0.97c
Explant Mean Values	0.88a	0.82b	

Values are means of 50 observations per explant per treatment.

Number of shoots regenerated from explants

As cytokinins and auxins are the main growth hormones required for *in vitro* plant regeneration; hence, the regeneration medium comprised of MS salts supplemented with diverse combinations of BAP, IAA, NAA and KN. The regeneration initiated 2-3 weeks after culture. Organogenesis started from the calli of both explants on all regeneration medium combinations. While comparing both the explants, leaf calli produced more shoots than intermodal stem segments calli (Table 5). Total number of shoots per explant was counted and efficiency of medium was evaluated. Leaf explants produced 2.87 shoots per callus as against 1.35 shoots from each callus piece induced from intermodal stem segments. RM_2(MS + 2 mg L^{-1}BAP + 2.5 mg L^{-1}NAA) was observed to the best medium for getting a large number of shoots (5.00) compared to other regeneration media. Whereas, the lowest number of shoots per callus (1.56) were induced on RM_4 (MS + 0.5 mg L^{-1}BAP + 2 mg L^{-1}NAA) from leaf explants. Stem internodal segments produced maximum shoots (2.6) on RM_2 and the lowest number of shoots (0.5) was produced from each callus piece on RM_4.

Plant Regeneration Frequency as affected by regeneration media

Regeneration frequency from both the explants was examined on different regeneration media. While comparing both the explants, leaves showed more regeneration frequency than intermodal stem segments (Table 6). Leaf explants showed 64.99% regeneration frequency as against 35.00% of intermodal stem segments. Regeneration frequency response was the maximum (64.99%) at RM_2. Whereas, the lowest regeneration frequency (33%) was observed at RM_6 from leaf explants. Internodal stem explants produced maximum callus (35%) at RM_2 and the lowest callus (15.50%) was observed on RM_6.

Rooting frequency

The regenerated shoots from leaf and stem-derived calli were shifted to root induction medium (MS). Majority of shoots developed roots. Higher rooting response (96%) was observed in the shoots originating from leaf callus as against 70% in shoots derived from stem callus. Rooted plants were ready for hardening in pots within 10–12 weeks of culture.

Table 5. Number of shoots for leaf and stem explants of potato *cv.* SH-5 as influenced by different regeneration media

Plant growth regulator supplement (mg L^{-1})				**Number of shoots per explant**		**Treatment Mean Values**
				Leaf	Internodal Segments	
BAP	NAA	IAA	KN			
0	0	-	-	0.000i	0.000i	0.000i
2	2.5	-	-	5.00a	2.60d	3.80a
2	0.5	-	-	4.30b	2.10e	3.20a
0.5	2	-	-	1.56f	0.50h	1.03c
2	-	0.5	-	0.89c	1.70f	2.79b
-	0.5	-	4	2.50d	1.20g	1.85b
	2.87a	1.35 b	-			

Values are means of 50 observations per explant per treatment, where 6-benzylamino purine (BAP), 1-Naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and Kinetin (KN)

Table 6. Regeneration frequency of potato cv. SH-5 as influenced by different regeneration media

Plant growth regulator supplement (mg L^{-1})				**Regeneration Frequency**		**Treatment Mean Values**
				Leaf Callus	Stem Callus	
BAP	NAA	IAA	KN			
0	0	-	-	0.000i	0.000i	0.000i
2	2.5	-	-	64.99 a	35.00d	49.99a
2	0.5	-	-	53.99b	26.00e	39.99b
0.5	2	-	-	26.00 e	11.25h	18.63e
2	-	0.5	-	40.99 c	21.75f	31.37c
-	0.5	-	4	33.00d	15.50g	24.25d

Values are means of 50 observations per explant per treatment, where 6-benzylamino purine (BAP), 1-Naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and Kinetin (KN)

Discussion

2,4-D has been proved as the most excellent auxin for callus initiation by several researchers (Khalafalla et al., 2010; Kaur et al., 2018; Jaffari et al., 2019). Hence our studies are in agreement with the earlier reports. Depending on 2, 4-D concentration there were

variable responses in percent callus induction, callus color, callus texture and degree of callus production. Initiation of callus could be observed from cut edges of both explants at all concentration of 2, 4-D. Similar results were reported by Fiegert et al., 2000; Jayasree et al., 2001 and Yasmin et al., 2003. The results showed that, when the MS media were supplemented with 4 to 8 mg L^{-1} of 2, 4-D, 100% of the explants formed yellow, and watery callus and recorded the highest degree (+++) for callus formation. These results are in proportion to Khalafalla et al. (2010) who obtained the same results. The results are also similar with the findings of Shirin et al. (2007) who recommended 3 mg L^{-1} 2, 4-D for callogenesis from internodal and leaf explants of potato.

The highest value for average callus weight was 0.88g from leaf explants as against 0.82g from intermodal stem segments of *cv*. SH-5. The maximum callus weight was attained (1.17g) and (1 g) at 2mg L^{-1} and mg L^{-1} of 2,4-D in leaf and internodal segments, respectively. The effect of altered 2,4-D concentrations and interaction effects of explants × concentrations showed significant differences on callus weight. Haque et al. (2009) reported the similar results where leaf callus weight was higher (0.86 g) followed by 0.82g and 0.77g from internode and nodal explants, respectively.

Regarding organogenesis, leaf and stem derived calli differed significantly in their regeneration response. Our study indicated higher regeneration efficiency (65%) from leaf-derived callus as against 35% from stem-derived calli. This is an indicative of the fact that leaf is more responsive to callus-plant regeneration than internodal stem explant in SH-5. Our result supported by Yasmin et al. (2003) who found that the leaf disc were always more responsive explant than internodal segments. Regeneration response of leaf and stem segments was also affected by the growth regulators used in the culture medium. RM_2 (MS + 2 mg L^{-1} BAP + 2.5 mg L^{-1} NAA) culture medium (define it) was selected to be the best medium for enhancing regeneration from leaf as well as internodal stem segment-derived calli. Here, again leaf-derived calli were more responsive on this medium than stem calli. Whereas, (RM_6 = 0.5 mg L^{-1}NAA) was the least regeneration responsive culture medium, with 35% and 15.5 % regeneration efficiency from leaf and stem-derived calli, respectively. Our results are in conformity with Yasmin et al. (2003), who also found the highest regeneration (80%) on MS + 2 mg L^{-1} BAP + 2.5 mg L^{-1} NAA followed by (70%) regeneration on MS + mg L^{-1} BAP + 1.25 mg L^{-1} NAA from leaf. Ali et al. (2006) also found the highest percent of shoot regeneration in the culture medium containing 1mg L^{-1}NAA and 4mg L^{-1}BAP and the lowest percent regeneration was observed on media containing 0.5mg L^{-1}BAP and 2mg L^{-1}NAA.

The different number of shoots/callus was observed on various regeneration media. The leaf explants produced 2.87 shoots per callus as against 1.35 shoots from each callus piece induced from intermodal stem segments. RM_2 (MS + 2 mg L^{-1}BAP + 2.5 mg L^{-1}NAA) was selected to be more suitable for getting a large number of shoots (5.00) than other regeneration media. Whereas, the lowest number of shoots per callus (1.56) was induced on RM_4 (MS + 0.5 mg L^{-1}BAP + 2 mg L^{-1}NAA) from leaf explants. Similar findings were

obtained by Rabbani et al. (2001) who obtained the highest number of shoots (14) per callus on medium having 2 mg L^{-1} BAP alone.

Majority of shoots produced roots when transferred to MS medium devoid of growth regulators (MSO). Higher rooting response (96%) was observed in the shoots originating from leaf callus as against 70% in shoots derived from stem callus. Anjum and Ali (2004) found the same results, describing maximum rooting frequency at cytokine free medium.

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