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Research Article

DIFFERENTIAL COMPETENCE FOR *IN VITRO* RAPID AND RELIABLE REGENERATION OF POTATO (*Solanum tuberosum* L.) CULTIVARS

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ABSTRACT. The present study tested *in-vitro* growth and performance of 14 exotic and local potatoes (*Solanum tuberosum* L.) cultivars under artificial long-day conditions. The study carried out in four steps; 1) effect of medium (ten nutrient medium) on *in vitro* plant growth, 2) callus induction and shoot proliferation from internode and leaf disc explant, 3) selection of nodal explants (apical, 1st and 2nd nodes) and position, 4) acclimatization and *ex-vitro* plant growth of regenerated explants. The nutrient growth medium were statistically significant difference ($P< 0.05$) on shoot length, number of leaf/shoot and tuber formation. The M7 (MS salts + B5 vitamins) medium was most suitable and efficient for *in vitro* regeneration. Internode explants of 14 potatoes cultivars demonstrated significant differences ($P < 0.05$) among them and showed superiority for calli induction compared to leaf disc explants. The redifferentiated from internode derived calli to adventitious shoot had positive responces on number of shoots (4.7) per plant of potato cv. Tokat-6/24 when subcultured on M7 medium containing 4 mg/l gibberellic acid, 0.5 mg/l 6-benzylaminopurine, 0.2 mg/l naphthalene acetic acid. The maximum (91.7%) acclimatization and survival rate of *in vitro* regenerated plantlets noted on peat moss: perlite: sand (1:1:1) when compared to other three potting mixture. The *in vitro* regenerated potato plants were vigorous, and produced healthy tubers in pots as well as field conditions. However, this protocol can be use for development of genetic engineer and large-scale production of healthy & disease-free potato seed tubers.

Keywords: *Apical-node, acclimatization, microtuber, shoot regeneration, substrates*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth major economic crop after rice, wheat, and maize with an annual production of 388 million tons in the world [1]. It mainly cultivated as a food crop and secures second place in human food consumption in the world. It provides a substantial source of carbohydrates, starch, minerals, vitamins, protein, and free amino acids especially lysine that is lacking in cereals crops [2, 3].

Although, potato seed tubers are expensive, therefore, half of the total expenditure of crop is spent for purchasing the quality seeds. However, most of the tuber seeds for potato production are uncertified, degraded, and infected with various pathogens [4]. Besides, the production of elite potato seeds through breeding can take long years and is expensive [2]. Plant biotechnology-based tissue culture techniques are the most powerful tools for

the producing quality seeds and incorporation of desired gene for crop improvement in the modern era [5, 6, 7]. However, an efficient plant regeneration depends on plant growth regulators (PGRs) combination, the genetic background of the donor plant or seeds, whereas, external physical parameters like light intensity, temperature and culture conditions play major role [8]. The physical parameters contribute to accelerating the multiplication of growing plantlets throughout the year without depending on the season. Besides this, tissue culture techniques is a prerequisite for genetic transformation and genome editing and disease free microtubers production [6, 9] to increase yield and quality characteristics of potato [10]. The genetically modified potato lines resistant to insects, virus, nematode, herbicides, salt, drought and cold can be used in future breeding programs [11].

The previous studies showed dedifferentiation and subsequent redifferentiation from callus were influenced by many factors including different potato explants, like leaves, meristems, tuber discs, petioles with intact leaflets, nodal or internodal segments and differences among the potato cultivars [12]. Therefore, shoot initiation and multiplication through either indirect or direct organogenesis is highly genotype-dependent [13]. Thus, the identification of appropriate media combinations and culture-responsive genotype is a prerequisite for the development of an efficient genetic transformation protocol. Therefore, present study was to compare ten basal nutrient media on 14 local and exotic potatoes cultivars and their nodal junction (positions) explants to select a highly efficient and reliable regeneration protocol for genetic transformation and genome editing. Thereafter, four substrates were compared for acclimatization and to select the best potting substrate for tissue culture-raised plantlets hardening and tuber production under glasshouse conditions.

MATERIALS AND METHODS

Plant Material

In the present study, 14 local and exotic potato cultivars namely; 1. Tokat-10/1, 2. Tokat-6/24, 3. Tokat-BCB, 4. Tokat-13/8, 5. Tokat-3/161, 6. Marabel, 7. Innovator, 8. Vangogh, 9. Morfana, 10. Hermes, 11. Granola, 12. Agata, 13. Agria, and 14. Lady Claire were tested for *in vitro* culture responses.

Surface (Tuber and Shoot Tips) Sterilization

Tubers of all cultivars were surface sterilized with 5% sodium hypochlorite (commercial bleach) for 20 minutes and then rinsed 3 times for 5 min with sterilized water. Subsequently, each potato tuber was covered with a brown paper bag and kept at 20 ± 1 °C in dark condition for five weeks for rapid sprout initiation. After five weeks, sprouts were excised and surface sterilized with 2.5% sodium hypochlorite (v/v) again and then rinsed 3 times for 5 min with sterilized water.

Culture Condition

For all the culture medium pH were adjusted to 5.6-5.8 with 1N HCl, and then autoclaved (121 °C and 1.5 kg/cm pressure) for 20 min. In addition, all plant growth regulator solutions were sterile filtered through a double 0.2 µm filter. The culture vessels were kept at 25±1 °C with a 16/8 h photosynthetic photon flux (PPF) light and dark photoperiod respectively. Light intensity was supplied at 30 µmol $m^{-2}s^{-1}$ by cool-white

fluorescent lamps. The plant growth regulators, gelrite and gellan gum was purchased from Sigma-Aldrich Co, St. Louis MO, and Duchefa Biochemie B.V, Haarlem, Netherlands.

Methods for Different Media Preparation and Explant Selection

The procedure was divided into four steps: 1. Selection of the basal nutrient medium, 2. *In vitro* selection of best responsive potato cultivar, 3. Selection of suitable culture explant (node position) and PGRs combinations, 4. Selection of best acclimatization and hardening substrate for *in vitro* regenerated plant.

Selection and Optimization of The Basal Nutrient Medium

After surface sterilization, internode explants of potato cv. Tokat-1/10 was cultured on gelrite solidified media; M1 = MS medium (1/2 concentrations of NH_4NO_3 & KNO_3), M2 = SH Medium, M3 = MS salts plus Nitsch vitamins, M4 = 1/2 MS Macro salts plus full vitamins, M5 = MS (NH_4NO_3 replaced by NaNO3), M6 = Gamborg (B_5) medium, M7 = MS salts + B_5 vitamins, M8 = MS Medium, M9 = Chu Medium (N_6), M10 = MS medium without vitamins and supplemented with 3% sucrose (w/v) for identification of the best multiplication media. The Tokat-1/10 was a new released and popular potato cultivar and its performance under the *in vitro* culture condition was better [6]. Therefore, it was used for medium selection in the present study. All the culture media contained 8-10 explants that were replicated thrice. To identify the best medium for explant growth (multiplication), root and shoot induction (percentage), shoot length, number of leaf per plant, leaf length, leaf width, tuber formation, and number of tubers per plant were recorded after 5 weeks of culture initiation. The out-performing medium M7 (MS salts + B5 medium) was selected for next experiment.

Selection of Best Potato Cultivars Through Callus and Adventitious Shoot Regeneration

To identify high frequency callus induction and adventitious shoot regeneration from 14 potato cultivars, leaf disc and internode explants were cultured on M7 medium (MS salt + B5 vitamins; selected from 1st step of media optimization) supplemented with 4 mg/l gibberellic acid (GA_3), 0.5 mg/l 6-benzylaminopurine (BAP), 0.2 mg/l naphthalene acetic acid (NAA), 3% sucrose (w/v) and solidified with 0.3 % (w/v) gelrite. Based on callus induction (%), adventitious shoot regeneration (%) and the number of shoot per explant were recorded after five weeks of culture and out-performed cultivar was selected for further experiments.

Selection of Explant (Nodal Position) and PGRs Combinations

The M7 medium (MS salts + B5 vitamins) and Tokat-6/24 potato cultivar was used for identification of suitable explant (nodal position) and growth regulator combination for maximizing *in vitro* growth and regeneration. Therefore, two weeks old *in vitro* grown seedlings of Tokat-6/24 were used for 1st, 2nd and apical nodal explants were cultured on M7 medium containing constant concentration of 4 mg/l GA_3, 0.2 mg/l NAA and variable concentrations of 0.50, 0.75 and 1.0 mg/l BAP. The culture medium also supplemented with 3% (w/v) sucrose and solidified with 0.3% gelrite. The data on shoot induction (%), number of shoots/explant, shoot length (cm), leaf length (mm), leaf width (mm), tuber

induction (%), number of tubers/plant, and root induction (%) were recorded after five weeks of culture inoculation.

Acclimatization and Hardening

The healthy and well-rooted plantlets of 15-20 cm long were taken from *in-vitro* culture and then washed in running tap water thoroughly to remove medium adhering to roots. These plantlets were transferred to four potting substrates containing peat moss (100%), perlite (100%), peat moss: perlite: soil (1:1:1, w/w), and peat moss: perlite: sand (1:1:1, w/w) for acclimatization.

The soil used in the experiment had 20% (w/w) sand with 53% water saturation percentage, 0.04 % (w/w) total salts, pH of 7.56, lime, 137.3 kg/ha phosphorus, potassium of 1,746.7 kg/ha, organic matter of 1.34 % (w/w), total nitrogen of 0.07 % and organic carbon of 0.78 %. Peat moss had a porosity of about (68 % v/w), pH 6.0 and EC of 0.1 dS m^{-1}) which allows for high water absorption capacity. The perlite used in the acclimatization experiment had a bulk density of about 50 kg/m and contained SiO_2, K_2O, Fe_2O_3, MgO, Al_2O_3, Na_2O, and CaO.

Each pot was covered with transparent polyethylene bags to create high relative humidity. After two weeks, the bags were gradually opened to avoid heat shocks from the atmosphere. The acclimatized plants were maintained and hardened under ambient daylight conditions at 23 ± 2 ^oC in the glasshouse.

Statistical Analysis

The data was subjected to one-way analysis of variance (ANOVA, IBM® SPSS® statistics 24.0 for Windows), and the post hoc tests were performed using Duncan's test. The treatments were arranged in a completely randomized blocks design with three replications. The data given in percentages were subjected to arcsine (√X) transformation [14] before carrying out statistical analyses.

RESULTS AND DISCUSSION

In the present study, sprouts/eyes rapidly developed from seed tubers when wrapped in dark brown paper bags and stored at 20 ± 1 °C with 80% relative humidity after surface sterilization. After five weeks, sprouts of about 8-10 cm length were cut from the mother tubers and again surface sterilized by 2.5% sodium hypochlorite for 20 min and subsequently rinsed with sterilized double distilled water. Thereafter, these sterilized plantlets were used as explant sources for further experiments.

Selection and Optimization of The Basal Nutrient Medium

In the present study, internode explants of potato cv. Tokat-1/10 was cultured on ten different nutrient basal medium devoid of growth regulators Table 1 for identification of appropriate nutrient basal medium for further research. The results showed the statistically significant interactions ($P \leq 0.05$) between basal nutrients medium and internode explants on shoot length, number of leaf /plant, leaf length, leaf width, tuber formation, and number of tuber per plant Table 1. Whereas, shoot and root induction percentages were exhibited non-significant interaction with used media that showed cent percent root and shoot induction in the all nutrient media. Among the used media, the M7 medium (MS salt with B5 vitamin) showed superiority and highest shoot length (44.0

cm), tuber formation (100%), and maximum number of tuber (2.2) per plant (Fig. 1a). The results exhibited maximum number of leaves per plant (11.4) was noted on M1 medium that was derived from MS medium containing 1/2* NH_4NO_3 & KNO_3 concentration without plant growth hormones (Fig. 1b). Whereas, M9 medium (Chu) showed the maximum leaf length and width, respectively, 10.3 mm and 5.3 mm (Fig. 1c) growth as compared to the other mediums. The color of the regenerated leaf on M7 medium was darker green compared to M1 and M3 medium regenerated leaf on internode explants. The leaf regenerated via internode-derived explant on M1 and M3 medium exhibited yellowish light green leaf color, week and thin morphology (Fig. 1d).

The present results illustrated that different nutrient media significantly affect tuber formation and number of tubers/plant that ranged 33.3 - 100% and 0.3 - 2.2 respectively. Among the tested media, MS salt plus B5 vitamin (M7) medium performed excellent and produce 100% tuber with the maximum number of tubers (2.2) per plant. Overall comparison of the medium indicated that M7 medium was the best medium for rapid growth from the internode junction and showed balanced nutritional composition for the potato explants growth in culture condition.

Table 1. *Effect of different nutrient basal medium on in-vitro plant regeneration from internode explants of Potato cv. Tokat-1/10 after 5 weeks of culture inoculation*

Media[#]	Shoot induction (%)	Root Induction (%)	Shoot length (cm)*	Number of leaf / plant		Leaf length (mm)		Leaf width (mm)		Tuber formation (%)		Number of tubers per shoot/ plantlet	
M1	100	100	16.9±3.6	cd	11.4±1.5 A	5.9±1.2	bc	3.9±0.3	abc	73.3	ab	1.1±0.8	b
M2	100	100	12.5±1.8	de	8.6±2.0 abc	7.1±1.6	b	4.4±1.8	abc	46.7	b	0.6±0.3	b
M3	100	100	13.6±2.0	de	5.9±1.4 bcde	5.7±1.5	bc	3.5±0.2	abcd	20.0	b	0.3±0.1	b
M4	100	100	14.6±1.4	de	4.7±1.2 de	4.5±0.2	bcd	4.5±0.3	ab	60.0	ab	0.8±0.4	b
M5	100	100	15.5±2.9	de	7.1±3.0 bcd	2.7±1.0	d	2.7±0.9	bcd	53.3	ab	0.7±0.8	b
M6	100	100	9.9±0.3	e	4.8±2.4 de	2.7±1.4	d	2.3±1.9	cd	60.0	ab	0.7±0.5	b
M7	100	100	44.0±0.9	a	5.3±2.4 cde	5.5±2.4	bcd	3.6±1.7	abcd	100.0	a	2.2±0.7	a
M8	100	100	21.1±6.1	c	5.9±0.9 bcde	2.9±0.8	d	1.6±0.2	d	46.7	b	0.7±0.3	b
M9	100	100	35.3±2.9	b	8.9±1.4 ab	10.3±1.8	a	5.3±0.8	a	73.3	ab	1.3±1.0	ab
M10	100	100	20.8±3.1	c	2.4±0.7 E	3.5±1.6	cd	1.7±0.6	d	33.3	b	0.3±0.1	b

*Values with in a column followed by different small letters are significantly different ($p<0.05$) using Duncan's multiple test. [#] M1 = MS medium (1/2 concentrations of NH_4NO_3 & KNO_3), M2 = SH Medium, M3 = MS salts + Nitsch vitamins, M4 = 1/2 MS Macro salts + full vitamins, M5 = MS (NH_4NO_3 replaced by NaNO3), M6 = Gamborg (B_5) medium, M7 = MS salts + B_5 vitamins, M8 = MS Medium, M9 = Chu Medium (N_6) and M10 = MS medium.

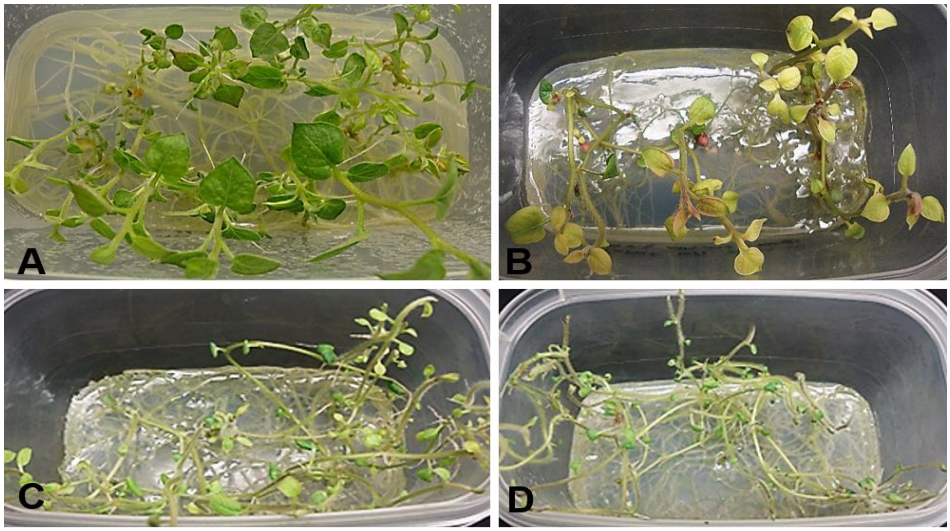

Fig. 1. *Shoot growth from internode explants of Tokat-1/10 cultivar after 5 weeks of culture in different nutrient basal media A) M7 medium (MS salts with Gamborg B_5 vitamins), B) M1 medium (MS 1/2*NH_4NO_3 & KNO_3), C)* M3 medium (*MS Medium with Nitsch vitamins), D) M9 medium (N_6 Chu medium)*

The biotechnology based approaches especially plant tissue culture is main contributor for crop improvements in the term of virus-free plant, introgression of insect resistance gene through genetic transformation. Therefore, rapid and reliable regeneration protocol is prerequisite [6]. For potato tissue culture protocol, the present study investigated effects of different media, cultivars, different position of nodal explant, combinations of growth regulators and their positive and negative interactions on callus and adventitious shoot induction. The present results showed the statistically significant interactions ($P \leq 0.05$) and pronounced variation between basal nutrients medium and internode explants on shoot length, number of leaf / plant, leaf length, leaf width, tuber formation, and number of tuber per plant. Although, axillary shoots and leaf disc explants are commonly used for indirect (via callus) and direct (adventitious shoot) *in vitro* shoot multiplication of potato cultivars [13, 15]. The M7 medium derived plant were more vigorous and healthy, whereas, tuber formation and size was also positively affected by medium, which showed a similar result as in previous studies [16, 17]. The researchers also emphasized that combination of MS salt and B5 vitamins enhanced the growth of the regenerated potatoes plant in culture medium. The present research also emphasized that Chu medium (M9) had the maximum leaf length and width (Fig. 1c) growth as compared to the other mediums are agreement with Kumar et al. [18] and Padilha et al. [19]. The researchers identified that concentration of ammonium was crucial for the plant tissue and organ growth in the culture medium and had positive impact on plant growth.

Among the tested media, M7 medium performed better and produce 100% tuber with the maximum number of tubers (2.2) per plant are agreement with Naqvi et al. [16]. The authors revealed that many growth factors significantly affect the tuber formation and growth in culture condition. Apart from this, genetic structure and parental pedigree also play the vital role in the tuber formation in the culture condition [20]. As indicated in the present results, M7 medium was the best medium for overall growth from the internode junction that provide sufficient nutrient component for plant growth are agreement with Saker et al. [15]. They emphasized that proper and balanced nutritional composition of the medium must be improved the plant growth in culture condition.

In vitro selection of potato cultivars

In the 2nd step of this study, leaf discs and internode explants of 14 potato cultivars were cultured on M7 medium supplemented with 4 mg/l GA_3, 0.5 mg/l BAP, 0.2 mg/l NAA and 3% sucrose (w/v) for callus and adventitious shoot induction Table 2. All the potato cultivars induced cent percent callus on internode explant, whereas it was ranged 33.3 - 100% on leaf discs explant. The minimum callus induction on leaf disc explant were ranged 33.3% and 40% on cv. Lady Claire and Tokat-3/161 respectively.

It was observed that during the first week of explant inoculation, leaf discs showed swelling at cutting edges and calli initiation was started at the lower side adjacent to medium. Whereas, internode explant showed enlargement and slight proliferation of callus formation at the wounded edges, but they were variably different in color, texture and biomass of callus (Fig. 2). The calli formation continued to proliferate on internodes explant of all the cultivars especially at the cutting edges that were adjacent to medium are agreement with Saker et al. [15]. They recorded variable morphogenic callus induction responses on leaves, internodes, and tuber explants of potato cv. Desireé when cultured on different regeneration media. Apart from this, many factors affected regeneration and callus induction ability *in vitro* conditions of potato cultivars [21].

Table 2. *Callus induction and adventitious shoot regeneration on leaf discs and internode explants cultured on M7 medium supplemented with 4 mg/l GA_3, 0.5 mg/l BAP and 0.2 mg/l NAA of 14 potato cultivars*

Potato Varieties	Callus induction (%)		Adventitious shoot regeneration (%)		Number of shoot per explant	
	Leaf disc*	Internode*	Leaf disc*	Internod*	Leaf disc*	Internode*
Marabel	100 a	100.0	60.0 a	100 a	4.1 a	3.2 b
Innovator	100 a	100.0	53.3 a	6.7 bc	1.9 bcde	2.8 bc
Tokat-10/1	100 a	100.0	43.3 abc	26.7 b	3.0 b	1.7 bc
Tokat-6/24	100 a	100.0	46.7 ab	100 a	2.3 bcd	4.7 a
Vangogh	100 a	100.0	53.3 a	16.7 bc	2.4 bc	1.9 bc
Agata	100 a	100.0	46.7 ab	13.3 bc	1.5 cdef	1.8 bc
Tokat BCB	100 a	100.0	10.0 bc	23.3 bc	0.7 fg	1.7 bc
Tokat-13/8	100 a	100.0	10.0 bc	23.3 bc	0.7 fg	1.8 bc
Tokat-/161	40.0 b	100.0	0.0 c	0.0 c	0.0 g	0.0 d
Granola	100 a	100.0	0.0 c	0.0 c	0.0 g	0.0 d
Hermes	100 a	100.0	3.3 c	0.0 c	0.3 fg	0.0 d
Lady Claire	33.3 b	100.0	3.3 c	6.7 bc	0.3 fg	0.3 c
Marfona	100 a	100.0	10.0 bc	13.3 bc	1.2 defg	1.7 c
Agria	100 a	100.0	33.3 abc	13.3 bc	1.0 efg	1.7 c

*Values with in a column followed by different letters are significantly different ($p<0.05$) using Duncan's multiple test.

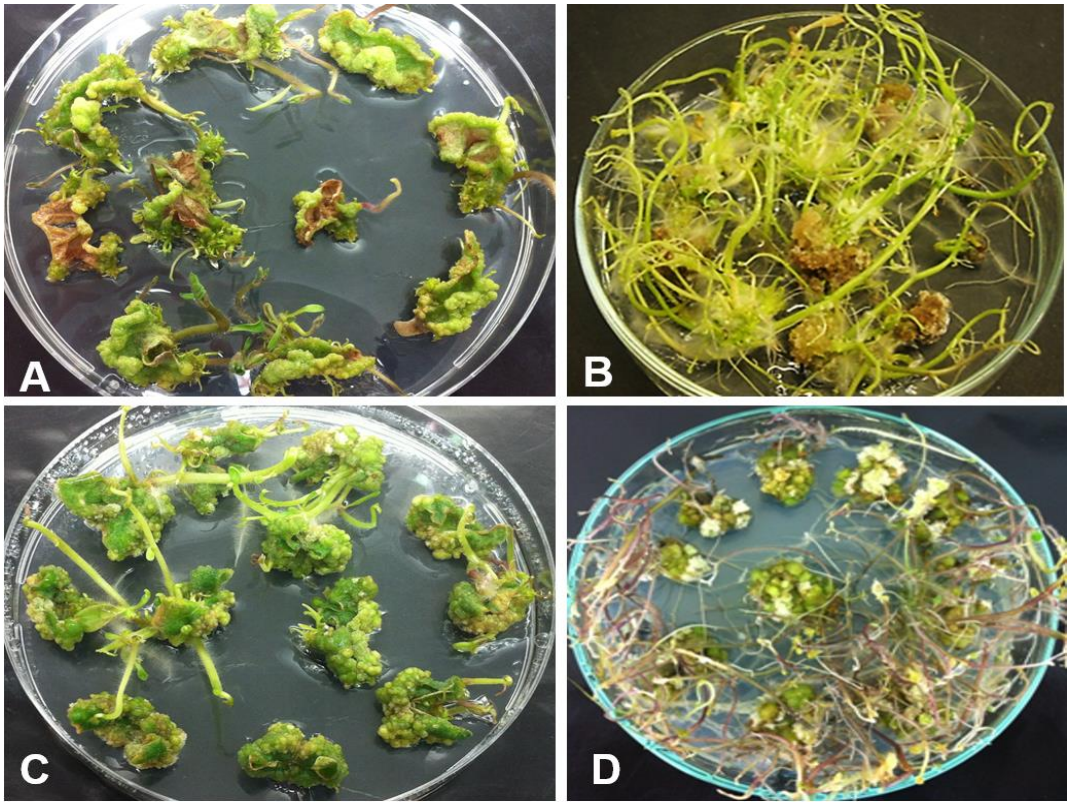

Fig. 2. *Callus induction and adventitious shoot initiation on M7 medium supplemented with 4 mg/l GA_3, 0.5 mg/l BAP and 0.2 mg/l NAA and from leaf and internode explants after 4 weeks of culture. A and B) leaf and nodal explants of cv. Marabel, C and D) leaf and nodal explants of cv. Tokat 6/24*

In the present study, the adventitious shoot regeneration was statistically significant ($P \leq 0.05$) among the potato cultivars, respectively, ranged 3.3 - 60% and 6.7 - 100% on leaf disc and internode explants Table 2. Two cultivars named Tokat-3/161 and Granola could not regenerate adventitious shoot in the M7 medium and showed recalcitrant nature in culture conditions. Previous studies also emphasized that it is very challenging to obtain a genotype-independent protocol for inducing adventitious shoots regeneration under culture conditions. Because the process of organogenesis/shoot initiation from different potato explants may vary among cultivars and especially when it depends on the donor plants [15, 22]. This study showed a high capacity for adventitious shoot regeneration from leaf disc and nodal junction explant respectively in potato cultivars Marabel and Tokat-6/24 which is similar with Thiruvengadam et al. [23], Rohela et al. [24]. Rohela et al. [24] pointed out the genotypic difference for regeneration capabilities of different explant during the in vitro culture conditions. This may be due to their morphological differences and variability in tissues (leaf disc and internodes) of the used explant [22]. In the present study, the number of shoots per explant was statistically significant different ($p \leq 0.05$) among the potato cultivar that varied between 0.3 - 4.1 and 0.3 – 4.7 shoots/explant on leaf disc and nodal junction, respectively Table 2. While the maximum number of shoots per explant was seen (4.1 shoots) on the leaf explants of the Marabel, and it was 4.7 shoots on the internode explants of the Tokat 6/24 cultivar. The induction of number shoot per explant was better on internode explant as compare with leaf disc explant of the most potato cutivars. The potato cv. Tokat-3/161 and Granola did not respond well and failed to regenerate a single shoot in culture condition. In the similar context, previous reports highlighted that the de novo organ formation and shoots induction from different origins explants i.e leaf, apical shoot, root, hypocotyl, and internode of potato cultivars [25, 26]. It is well documented that the genetic background of the genotype, origin, and type of the cultured explant and culture medium always affect the growth of cultured explant either in positive or negative manner [26].

Selection of explant (nodal position) and PGRs combinations

In the 3rd step, *in vitro* effect on morphological responses of M7 medium (MS salts with B5 vitamins) supplemented with 4 mg/l GA_3, 0.2 mg/l NAA and variable concentrations (0.50, 0.75 and 1.0 mg/l) of BAP on different nodal position (1^{st} and 2^{nd} nodes from above and apical node) explants of potato cv. Tokat-6/24 were investigated. The nodal position significantly affected the plant growth in culture and variable regeneration responses were recorded when BAP concentration was changed in the culture medium Table 3. The cent percent callus induction was observed in all explants and, therefore callus induction data was not given in Table 3. The nutrient medium supplemented with GA_3, BAP and NAA enhanced shoot induction, number of shoot per plant, leaf length and width, tuber formation, number of tuber/plant, and root induction on internodes, especially on apical node. The shoot induction was ranged from 75 to 100% among the media containing different BAP concentrations. Whereas, average shoot induction ranged from 80.6 to 100% on different nodal position are agreement with Mishra & Pathak [27]. The researchers emphasized that nodal position and season highly influenced in vitro shoot induction and proliferation in *Emblica officinalis* Gaertn. They further stated that physiological condition of mother plant while explant excision must be influence the culture response during the explant growth in culture codition. Moreover, it is generally belive that actively growing shoots or apical shoot performed well and gave excellent results in culture [28].

Table 3. *Effect on plant regeneration and tuber fromation of nodal position and apical node explants when cultured on M7 medium supplemented with 4 mg/l GA_3, 0.5-1 mg/l BAP and 0.2 mg/l NAA of cv. Tokat-6/24 after 5 weeks of culture inoculation*

Explant	**BAP**			**Average**
Shoot induction (%)	**0.50 mg/l**	**0.75 mg/l**	**1.0 mg/l**	
Apical node	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	**100.0a**
2nd node	75.0±25.0 c	91.7±14.4 b	91.7±14.4 b	**86.1b**
1st node	75.0±25.0 c	75.0±25.0 c	91.7±14.4 b	**80.6b**
Number of shoot / plant				
Apical node	5.3±1.0 bc	5.7±0.3 b	6.9 ±0.4 a	**5.96a**
2nd node	4.5±0.6 c	4.7±0.6 bc	5.3±0.6 b	**4.83b**
1st node	4.3±0.6 c	4.3±1.0 c	5.0 ±0.6 bc	**4.53b**
Shoot length (cm)				
Apical node	8.2±0.7 b	8.8±0.8 b	9.4±0.5 a	**8.8a**
2nd node	5.7±0.4 d	5.5±0.d	6.3±1.0 c	**5.8b**
1st node	6.2±0.8 c	5.1±0.1 d	5.2±1.0 d	**5.5b**
Leaf length (mm)				
Apical node	2.9±0.9 c	3.7±1.6 b	6.1±0.4 a	**4.2a**
2nd node	0.8±0.4 d	1.4±0.8 d	2.1±0.4 cd	**1.4b**
1st node	1.7±0.5 d	0.9±0.4 d	2.6±1.3 b	**1.7b**
Leaf width (mm)				
Apical node	2.0±0.5 a	2.2±0.1 a	3.0±0.3 a	**2.4a**
2nd node	1.3±0.4 ab	1.6±0.3 b	1.9±0.3 b	**1.6b**
1st node	0.8±0.3 c	1.4±0.1 b	1.2±0.6 bc	**1.1b**
Microtuber induction (%)				
Apical node	58.3±28.9 c	66.7±14.4 b	100.0±0.0 a	**75.0a**
2nd node	58.3±14.4 c	58.3±14.4 c	83.3±14.4 ab	**68.3a**
1st node	33.3±14.4 cd	50.0±25.0 c	83.3±14.4 ab	**55.5b**
Number of microtubers/ plant				
Apical node	1.1±0.4 bc	1.6±0.6 b	2.8±0.3 a	**1.8a**
2nd node	0.8±0.1 c	0.9±0.2 bc	1.6±0.4 b	**1.1b**
1st node	0.6±0.1 c	1.0±0.3 c	1.0±0.3 c	**0.9b**
Root induction (%)				
Apical node	100±0.0 a	100.0±0.0 a	100±0.0 a	**100.0a**
2nd node	75.0±5.0 c	75.0±5.0 c	91.7±4.4 b	**80.5b**
1st node	45.0±2.8 d	75.0±5.0 c	91.7±4.4 b	**70.6b**

Note: Each morphological characteristic were evaluated separately for statistitical observation. Values with in a column followed by different letters are significantly different ($p<0.01$) using Duncan's multiple test.

The present results pointed out that the M7 medium with a combination of GA_3, NAA, and BAP was crucial and effective for the number of shoot regeneration that ranged from 4.53 to 5.96 on different nodal positions. The maximum number of shoot per explant was observed on M7 medium supplemented with 4 mg/l GA_3, 0.2 mg/l NAA and 1 mg/l BAP (6.9 shoots), and followed by 4 mg/l GA_3, 0.2 mg/l NAA, 0.75 mg/l BAP (5.7 shoots) on apical node explants (Fig. 3a). The apical node explant showed superior and enhanced shoot growth per expalnt than the 1st and 3rd nodes of potato cv Tokat-6/24. The similar kind of growth trend was also observed for shoot length, leaf length and leaf width in this study Table 3. It is evident from the present results that nutrient medium (MS salts with B5 vitamins) supplemented with high concentrations of GA_3 and BAP along with the lower concentrations of NAA enhanced shoot induction with multiple shooting and increased shoot length, leaf length, and leaf width of regenerated plantlets are in agreement with Kumlay & Ercili [29]. They observed that the promotion of growth in terms of the number of shoots, shoot length, nodes and leaves was increased due to plasticity of the cell walls that lead to the hydrolysis of starch to sugars and lowered the water potential of the cells causing cell elongation in potato in vitro regenerated plant. In another report Dhital et al. [30] identified that internode explant showed a higher

frequency of shoot regeneration and the number of shoots/plants compared to leaf and petiole explants.

Although, it is evident that cytokinins are available with lower concentration in growing plant tissues particularly apical meristem or shoot tip [31, 32]. Therefore, most of the *in vitro* regenerated plants need a lower concentration of cytokinin but some explants need higher concentrations to enhance meristematic growth of culture explant [33, 34]. However, consideration must be given to the diversity of tissue types, which arrived or exist in potato tuber. It is, however, important that in potatoes, various tissues can be used as explants for shoot generation directly [22]. The present results also agree with Ghosh et al. [25], Kaur et al. [26], Kumlay & Ercisli [29], Harun-Or-Rashid et al. [35] and suggested that the utilization of hormones like BAP, NAA, IBA and IAA with GA_3 growth regulators positively affect regeneration and increased adventitious shoot formation on cultured explants of potato cultivars.

The present results showed microtuber induction and the number of microtubers per plantlet on different nodal explants ranged from 55.5 to 75% and 0.9 to 1.8 respectively Table 3. However, maximum microtuber induction and number were noted on M7 medium supplemented with 4 mg/l GA_3, 1.0 mg/l BAP and 0.2 mg/l NAA derived from the apical nodes. The shape of microtubers were round or elliptical with light green color agreement with Chen et al. [36]. They mentioned that induced microtubers under *in vitro* condition are green in color due to light-dark photoperiod, whereas, complete darkness produced microtubers are white or yellow in color. However, tissue culture conditions especially temperature (below 20°C) lowered the microtuber induction, number, and fresh weights of potato microtubers [15, 22].

In the present study, the maximum number of microtubers (2.8) was produced on a medium containing higher concentrations of GA_3 and BAP along with 3% sucrose concentration and these are in agreement with Saker et al. [15]. The researchers also obtained about 2.2 and 2.6 microtuber/explants in etiolated shoots of potato cv. Desirée when cultured on 3% sucrose in the growing medium. Whereas, Hossain et al. [37] recommended a higher concentration (8%) of sucrose for promoting microtuberization and size. The present results on root induction ranged from 70.6 to 100% on different nodal explants, whereas apical node was the best explant for rhizogenesis (Fig. 3b,c,d). The result is contradictory with Shahriyar et al. [38] who obtained a lower percentage of regenerated roots on potato in culture condition. But, it is well documented that a relatively lower concentration of auxin is required for rhizogenesis that enhanced the root induction in potato culture explants during the *in vitro* condition [15].

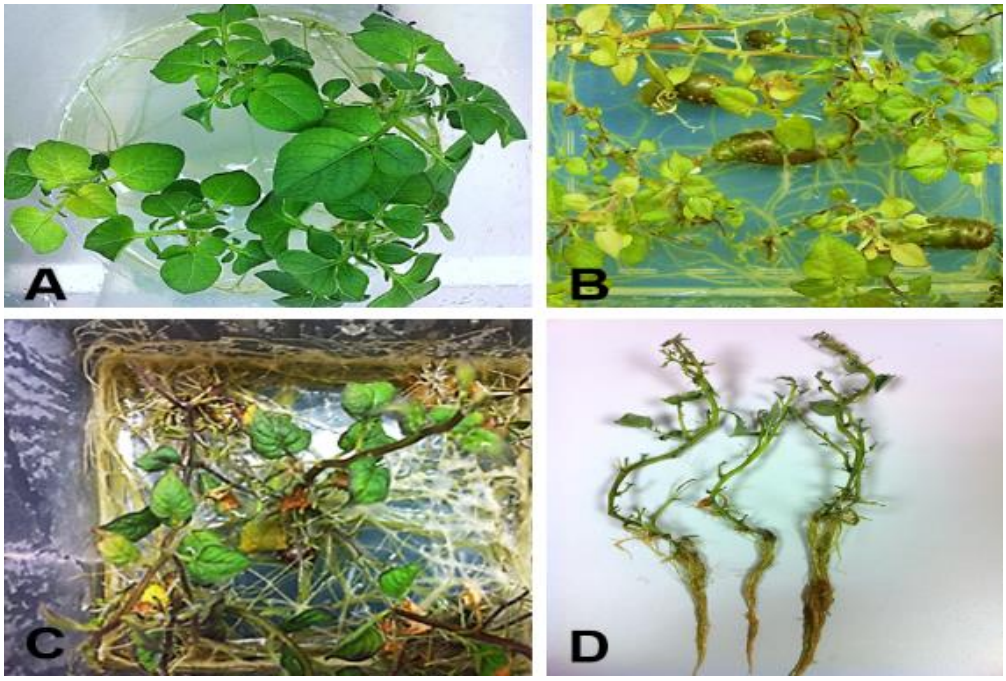

Fig. 3. *Plant regeneration from the apical node of cv. Tokat-6/24 on M7 medium supplemented with 4 mg/l GA_3, 1 mg/l BAP and 0.2 mg/l NAA after 5 weeks of culture. A) Shoot induction, B) micro tuber formation, C and D) root induction*

Acclimatization

The last leg of the experiment was to identify the best substrate type for *ex-vitro* acclimatization and hardening. The results showed statistically significant ($p \leq 0.05$) effects of substrate on hardening and acclimatization from apical node regenerated potato plantlets of cv. Tokat-6/24 Table 4. The plantlets transferred and acclimatized in all the potting mixtures (peat moss, perlite, peat moss: perlite: soil, peat moss: perlite: sand) were good for growth and showed variable acclimatization responses. The acclimatized plants percentages range from 41.7 to 91.7% and maximum acclimatization percentage was noted on peat moss: perlite: sand (1:1:1 ratio) substrate (Fig. 4b). The rest of the substrates demonstrated variable responses on growth inhibition and development, including chlorosis that affected acclimatization percentage (Fig. 4a,b,d). The peat moss: perlite: sand grown plants exhibited strong roots and thickness (0.8-1.0 mm) at their origin, which showed hair like structures at their end. The developing roots showed ramification and gentle curve while growing in peat moss: perlite: sand substrate. A similar type of root growth was not observed on other substrates.

Table 4. *Effect of various hardening substrates on ex-vitro acclimatization of in vitro regenerated potato plantlets*

Hardening substrates	Acclimatization (%)	Plant height (cm)	Number of node/plant	Number of leaf/ plant	Leaf length (cm)	Leaf width (cm)
Peat moss	41.7±2.88c	8.3±0.44bc	2.8±0.08b	3.2±0.30c	0.6±0.02b	0.4±0.02bc
Perlite	66.7±4.61b	12.8±0.53b	3.5±0.04b	3.8±0.20c	0.7±0.01b	0.6±0.01bc
Peat moss:perlite: soil	58.3 ±4.04b	19.1±0.53a	3.6 ±0.10b	6.9±0.40b	0.8±0.05b	0.8±0.06b
Peat moss: perlite: sand	91.7±6.35a	20.7±0.09a	5.7±0.19a	8.9±0.37a	1.3±0.08a	1.2±0.05a

Values shown in a column followed by different small letters are statistically different using Duncan's test at 0.05 level of significance

A pronounced feature of excellent acclimatization was the development of plant growth i.e. plant height, number of nodes, and leaf growth on above ground parts. The plants growing on peat moss: perlite: sand had maximum plant height (20.7 cm) that closely followed by peat moss: perlite: soil substrate. A number of nodes per plant were greatly affected by the type of substrates that ranged 2.8 – 5.7. Maximum nodes per plant were observed on plants hardened onto peat moss: perlite: sand substrate, whereas, the minimum was noted on peat moss. Nodes per plant on the rest of the substrates were statistically similar. The number of leaves per plant ranged 3.2 – 8.9 and the maximum number of leaf per plant was recorded on *in vitro* regenerated potato plants hardened on peat moss: perlite: sand. The number of leaf development on different substrates was significantly reduced and least growth was noted on peat moss substrate. The leaf length and leaf width ranged 0.6-1.3 cm and 0.4-1.2 cm respectively and showed the same growth phenology as the number of leaves per plant showed on substrates. Barpete et al. [39] reported that *in-vitro* regenerated plantlets of *L. sativus* showed individual variation in leaf morphology on different substrates. This may be due to differences in nutrients, water retaintion capacity, humidity, light exposure and other environmental factors which varied among growing substrates that affected growth of leaves.

Fig. 4. *Ex-vitro acclimatization of potato cv. Tokat-6/24: A) peat moos, B) peat moos-perlite-sand (1:1:1), C) perlite, D) peat-perlite-soil (1:1:1), E) acclimatized plant frown in filed condition, F) tuber formation*

It is well-established fact that the leaves of *in vitro* regenerated plants have poor water retention capacity due to the poorly functioning of stomata and high rate of water evaporation in newly regenerated potato plants [15]. Therefore, it is necessary that the new root system of the *in vitro* regenerated plants should start their function as soon as possible after the shifted in growing substrates. The early response of shifted plants may

compensate for the extensive water loss from the leaves. Therefore, growing roots have an essential role and specific function for uptaking water and nutrients for acclimatized potato plants [29, 39]. However, excellent growth in the potting mixture reflect the shoot/root length, as well as the number, is very important for the acclimatization and is helpful for nutrient and water uptake in potato plantlets [40]. All plants acclimatized in the *ex-vitro* conditions (glass-house) showed a high homogeneity and true to the type plant without any sign of morphological avoidance of somaclonal variation.

CONCLUSION

An efficient and reliable protocol is a prerequisite for the overall improvement of potato cultivars and the development of new potato lines through biotechnology based in vitro culture techniques. The regeneration and multiplication of shoots are also considered as a promoting step for utilizing the modern biotechnological approaches for genetic improvement of potato lines. Therefore, suitable medium, explants, PGRs, and cultivars identification should be the first step.

The present study suggested that the most favorable medium for regeneration from apical nodal junction explants was M7 (MS salts + B5 vitamins) medium containing higher concentrations of GA_3 (4 mg/l) and BAP (1.0 mg/l) along with the lower concentrations of NAA (0.2 mg/l) with 3% sucrose suitable for maximum shoot proliferation and regeneration of potato. The cv. Tokat-6/24 explants showed a better regeneration response than the other 13 potato cultivars and their apical nodal junction explants on shoot proliferation via complete regeneration. The acclimatization experiments revealed that peat moss: perlite: sand (1:1:1) improved the survival rate of shifted plantlets and is an excellent source for water and nutrient uptake. The protocol can be utilized for the development of transgenic potato lines through a biotechnological approach and further utilized for the large-scale production of healthy and disease-free potato seed tubers.

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