Pak. J. Bot., 48(1): 285-290, 2016.

ESTABLISHMENT OF AN EFFICIENT AND REPRODUCIBLE REGENERATION SYSTEM FOR POTATO CULTIVARS GROWN IN PAKISTAN

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Abstract

The present study was carried out to assess the effect of growth regulators in three different combinations on mass propagation of currently grown three potato cultivars cv. Desiree, Kuroda and Cardinal. Varietal response on *In vitro* regeneration under different hormonal combinations. For callus induction, internodes of potato cultivars were cultured on modified Murashige & Skoog (MS) medium, added with different growth hormonal combinations. Callusing frequency for all these treatments and cultivars were recorded and in callus induction medium (CIM1) explants showed significantly higher callus formation as compared to two other combinations. For shoot induction, calli were cultured on Murashige & Skoog (MS) modified medium, supplemented with different hormonal combinations. Shoot induction medium (SIM3) gave best shoot induction frequency as compared to other media combinations. On the same media, an average number of shoots per explant were obtained for cultivar Desiree which is significantly different from the other two media combinations. Overall, the *In vitro* regeneration and multiplication potential was highest in the variety Cardinal followed by Kuroda and Desiree. The interaction between different hormonal combinations and varietal response for all the parameters showed significant differences.

Key words: Potato; Regeneration; Tissue culture; Micropropagation.

Abbreviations: 2, 4-D: 2, 4-Dichlorophenoxyacetic acid, NAA: 1-naphthaleneacetic acid, BAP: 6-benzylaminopurine, ZR: Zeatin riboside, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid.

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important food crops grown world-wide. Potato production has reached over 300 million tons annually (Birch *et al.*, 2012). It is estimated that over 1 billion people use potato as a staple food. It stands fourth in most valuable food crops for human consumption in the world after wheat, rice and maize (Levy *et al.*, 2013). Globally, the tubers are considered as vital dietary source of starch, protein, antioxidants and vitamins, helping the plant both as a storage organ and a vegetative propagation system (Barrell *et al.*, 2013). In order to support the importance of potato, especially within the developing countries, the year 2008 was highlighted by the UN as the Year of potato (Levy & Veilleux, 2009).

For achieving rapid multiplication of plants and homogeneous material for horticulture industry, a well-established system has been used routinely known as micropropagation or plant tissue culture (Sabir *et al*., 2014). By using this technique, a single explant can be reproduced into several thousand plants in a minimum time. In order to exploit the potential of micropropagation for mass production of clonal plants, several commercial laboratories have been developed around the world in a comparatively short period of time since 1980s. Improvement of micropropagation protocols needs a precise process that commences with having the understanding of the plant species or genotype *in vivo* propagation features and also comprises the optimization of different chemical, physical and environmental elements for growth and multiplication during *In vitro* culture (Ruffoni & Savona, 2013).

Similarly keeping in view the importance of potato clones, tissue culture has been applied to get better potato production by means of micropropagation, pathogen free propagule development, and germplasm preservation (Bhuiyan, 2013). The regeneration of potato from tissue culture assists different purposes like micropropagation of exclusive plants, maintenance of hybridization potency, conservation and application of variants, and genetic transformation as well (Cingel *et al.*, 2010). High cost of tuber seed production, its long reproductive cycle that increases the possibility of viral and bacterial diseases is considered as one of the most important issue (Van *et al.*, 2012). To overcome the concerned problem, exploitation of tissue culture technique can enhance the yield and also minimize the costs of production by preventing diseases and use of pesticides (Alison *et al.*, 2011).

It is believed that chemical compositions of potatoes may also be changed by applications of certain growth regulators (Zahoor & Faheem, 2014). Another known influencing factor for *In vitro* organogenesis is that callus induction and regeneration ability is highly genotype dependent (Rani *et al.*, 2013). A very important biotechnological research objective is to produce callus rapidly in different potato cultivars (Sabeti *et al.*, 2013). However, in our conditions none of procedures have been reported which represents the suitable *In vitro* micropropagation for the potato cultivars grown in Pakistan.

This study was aimed to develop an improved/standard protocol allowing the micro-propagation of important potato cultivars currently grown in Pakistan which ultimately leads to mass propagation of healthy stock and successful *In vitro* seed tuber production.

Materials and Methods

The study was carried out in Agriculture Biotechnology Division NIBGE Faisalabad. Three cultivars namely Desiree, Kuroda and Cardinal were used for *In vitro* regeneration. 4-week old plants were used as explants for regeneration. Explants were arranged from internodal segments having size of almost 3 to 5 cm in length and these were used for callus induction and plant regeneration. All cultured explants were grown in modified MS medium to which different hormone combinations, sucrose and agar were added. The pH was adjusted to 5.8 before autoclaving at 121ºC and 15 lb psi for 20 min.

In this experiment 150 explants for each cultivar were used. The internodal explants were cultured on three different MS based callus induction media (Table 1) and placed in a growth room at 26°C ± 2ºC under low light intensity using 16 h photoperiod. After 3 to 4 weeks, depending on how prolific the callus growth was achieved, the explants with induced callus were shifted to shoot induction media (Table 2). After reaching an appropriate length, distinct green shoots were cut out and shifted to larger culture tubes (150 x 25 mm) containing root induction medium (Table 3).

Table 1. Combinations of various hormones used for callus induction in three different cultivars of potato.

Ingredients	CIM1 (Quantity per liter)	CIM2 (Quantity per liter)	CIM3 (Quantity per liter)
MS salt & B5	4.43 g	4.43 g	4.43 g
Zeatin Riboside	0.8 mg	-	-
2, 4-D	5.0 mg	-	-
BAP	-	2.0 mg	1 mg
GA3	-	2.0 mg	-
NAA	-	0.1 mg	1 mg
MSV1 vitamins	-	-	1 ml
JHMS vitamins	-	-	1 ml
Myo-inositol	-	-	100 mg

JHMS vitamins* - Folic acid (25 mg L^{-1}) + D-Biotin (5.0 mg L^{-1}) MSV1 vitamins*- Nicotinic acid (1.0 mg L^{-1}) + Pyridoxine HCl (1.0 mg L^{-1}) + Thiamine HCl (10 mg L^{-1}) + myo-inositol (100 mg L^{-1})

Table 2. Media combinations with hormonal supplements for subsequent regeneration of shoots.

Ingredients	SIM1 (Quantity per liter)	SIM2 (Quantity per liter)	SIM3 (Quantity per liter)
MS salt & B5	4.43 g	4.43 g	4.43 g
Zeatin Riboside	0.8 mg	-	3.0 mg
BAP	-	2.0 mg	-
GA3	0.1 mg	2.0 mg	-
IAA	-	-	1 mg
Myo-inositol	-	-	1 mg
3R vitamins	-	-	1 ml

3R (Vitamins)* Thiamine HCl (1 mg L^{-1}) + Nicotinic acid (0.5 mg L^{-1}) + Pyridoxine HCl (0.5 mg L^{-1})

Table 3. Rooting media for callus-derived shoots of different cultivars of potato.

Ingredients	RIM1 (Quantity per liter)	RIM2 (Quantity per liter)	RIM3 (Quantity per liter)
MS salt & B5	4.43 g	4.43 g	4.43 g
Zeatin Riboside	0.8 mg	-	-
BAP	-	2.0 mg	-
GA3	-	2.0 mg	-
NAA	-	0.1	1 mg
IBA	-	-	2 mg
MSV1 vitamins	-	-	1 ml
JHMS vitamins	-	-	1 ml
IAA	0.1 mg	-	-

Recorded parameters for Callus induction and Regeneration: The data were composed for the following parameters:

Percent callus induction: Callusing frequency (%) recorded by using formula:

$$\text{Callusing frequency (\%)} = \frac{\text{No. of explants producing callus}}{\text{No. of explants used}} \times 100$$

Regeneration frequency: Regeneration frequency recorded by using formula:

$$\text{Regeneration frequency (\%)} = \frac{\text{No. of shoots producing by callus}}{\text{No. of explants used}} \times 100$$

Rooting frequency: Recorded as number of roots divided by total number of shoots transferred to root induction media.

Statistical analysis: Experimental data were analyzed by applying Completely Randomized Design (CRD) with an arrangement of 150 replications per treatment per explant. The mean values were calculated for each treatment. In order to estimate the differences among treatments and genotypes, ANOVA with LSD test at 5% level of significance were statistically applied.

Results and Discussion

Several scientists have reported *In vitro* micropropagation from internodal explant of potato cultivars via "one step regeneration" system with a culture medium covering a blend of additional ingredients or "two stage regeneration" method with isolated cultural stages for callus production and root formation. Although, potato micropropagation has been reported by many scientists (Badoni & Chauhan, 2009; Rahman *et al.*, 2010; Yasmin *et al.*, 2011) but the potato regeneration is still considered as largely genotype dependent.

The results of callus induction experiments are shown in Fig. 1. The highest callus induction frequency was obtained in CIM1 for all the three cultivars tested, while poor response was obtained in CIM3. In CIM1, the highest callus induction frequency was obtained in Cardinal (78%) followed by Kuroda (70%) and Desiree (68%). In case of CIM2, Cardinal gave highest callus induction frequency (58%) followed by Kuroda (55%) and Desiree (42%). Similarly using CIM3 media, highest frequency of callus formation was again obtained in Cardinal (42%), followed by Desiree (40%) and Kuroda (38%). Results obtained during these experiments on callus proliferation in different hormone combinations were in line with the reported literature that auxin (2, 4-D) is necessary for the callus production (Majid *et al.*, 2014). In present study, 2, 4-D was found to have the most effective role on callus induction. Dagla, (2012) reported that the 2, 4-D and coconut milk both in combination had a significant effect on frequent growth of cultured potato tissues. Results showed that a soaring concentration of 2, 4-D up to 5.0 mg/L was most operative for callogenesis. These results also showed that the cultivars used were not significantly different in their response to callus induction using 2, 4-D.

The highest shoot regeneration frequency was obtained in SIM3 for all the cultivars tested while poor response was obtained in SIM2. In SIM1 media combination, no significant variation among the genotypes was observed where highest frequency of shoot regeneration was obtained in Cardinal (78%) followed by Kuroda (70%) and Desiree (65%). SIM2 hormone combination was found to be less responsive for shoot regeneration as it gave poor response in Cardinal (52%) followed by Kuroda (50%) and Desiree (40%). SIM3 gave significantly higher shoot regeneration efficiency compared to rest of the two media combinations but more or less similar for all genotypes tested, as Cardinal ranked first with 95% regeneration efficiency followed by Kuroda (90%) and Desiree (82%), respectively. These observations are in distinction to Khadiga *et al.* (2009) where they observed that media comprising NAA and BAP gave longest main shoot and highest node numbers. Apical dominance and the growth of lateral buds, stimulated by BAP while NAA drops single nodes growth and rooting of potato plantlets (Kumlay *et al.*, 2014). It is problematic to venture the reason for this difference but genotypic response could be one of the possible factors responsible.

The average number of shoots per explant were also recorded (Fig. 2). In SIM1, the highest shoot induction frequency was obtained in Desiree (6.3) followed by Kuroda (5.0) and Cardinal (4.9), respectively. For SIM2, Desiree ranked first having (5.0) shoots per explant followed by Cardinal (4.0) and Kuroda (2.1). Among all the genotypes, it was observed that SIM3 treatment gave the best results in Desiree (9.4), Cardinal (8.09) and Kuroda (6.3), respectively and also required less time for shoot induction. The results demonstrated that higher concentration of ZR (3.0 mg/L) in combination with IAA, myo-inositol and vitamins were more effective for shoot induction which is in agreement with the study reported by Stefan *et al.* (2001). This combination of growth hormones was best for shoot initiation and proliferation of potato *cvs* Desiree, Kuroda and Cardinal (Fig. 3). Use of different vitamins like JHMS and 3R vitamins could be the reason for prominent results from other growth regulators and confirmed the study reported by Jahn *et al.* (2011).

Moreover, in order to testify the results of all media combinations and cultivar response, number of roots per explant were also recorded (Fig 2). The highest number of roots formation per explant was obtained in Kuroda by using treatment RIM3 while poor response was obtained in Cardinal using RIM2. In RIM1, an average of 6.0 roots per explant obtained in Desiree followed by Kuroda (5.7) and Cardinal (3.9), respectively. RIM2 gave comparatively poor response as Desiree generated 5.2 roots followed by Kuroda and Cardinal having 2.8 roots, respectively. In RIM3, Kuroda ranked first for having 7.4 roots followed by Desiree (6.1) and Cardinal (4.8), respectively. Results regarding root formation were totally in contrast to Armin *et al.* (2011) in which they used different combinations of NAA and BAP for root induction of different potato cultivars. They observed that modified solid (MS) medium without NAA and BAP was considered as best for healthy roots in potato tissue culture. Our findings reveal that combinations of BAP and NAA with different hormones like Zeatin and IAA is responsible for the vigorous root formation.

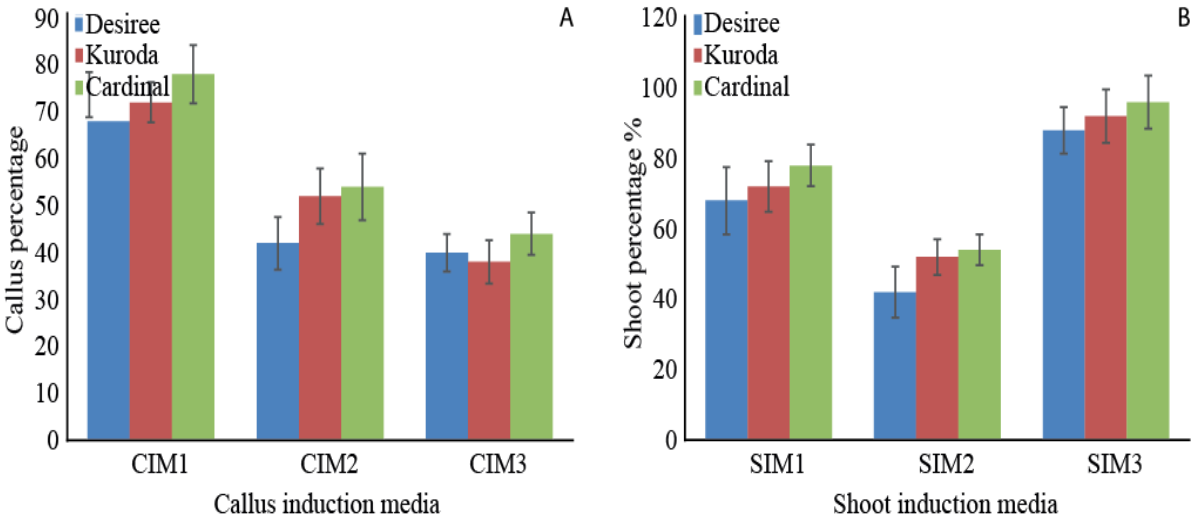

Fig. 1. (A) Percentage of callus production (B) Percentage of shoot induction on media (having different combinations of hormones) in three potato cultivars by using internodal explants. Average data was taken after 4 to 6 weeks on callus and shoot induction media.

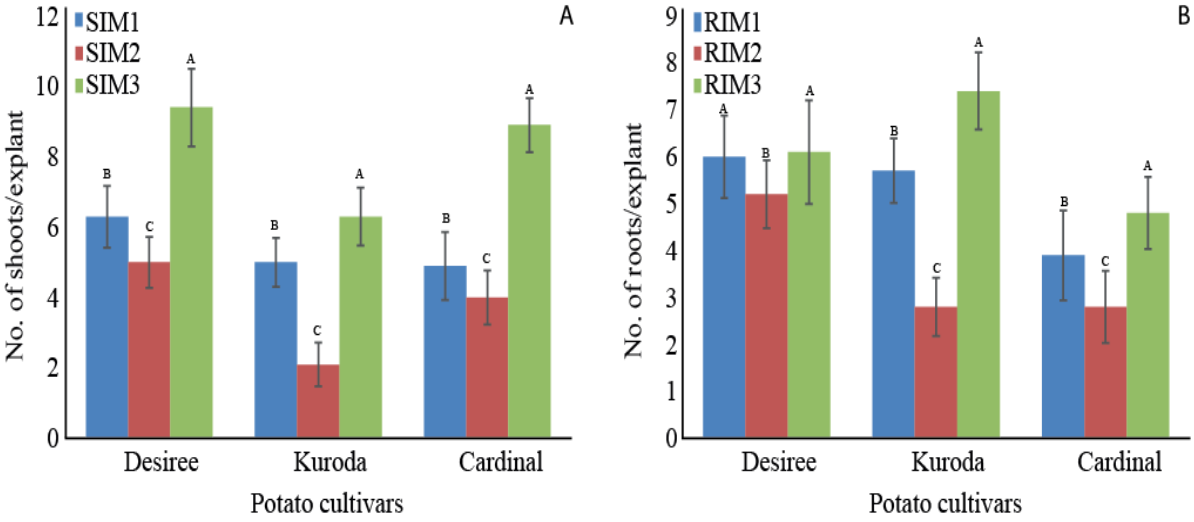

Fig. 2. (A) Number of shoots on three media combinations (B) Number of roots per regenerated shoot for three different potato cultivars with three media (having different combinations of hormones) were used as treatments. Values are means of 150 observations per treatment per explant.

Among three different rooting media combinations, RIM3 comparatively gave better results as it comprised of optimum concentration of NAA and IBA in combination with different vitamins (Table 3). It is frequently reported that a number of plant species including potato have genotypic dependent regeneration system (Danci *et al.*, 2008). Outcomes of present study with respect to influence of cultivar on micropropagation support the previous study reported by Thornton *et al.* (2013) that in potato micropropagation, a very important role is played by the genotype. For all the cultivars, SIM3 treatment gave better results for shoot regeneration. For other two cultivars in rest of the treatments Kuroda performed efficiently as compared to Desiree.

Conclusion

A protocol for the micropropagation of three potato cultivars grown in Pakistan i.e, Cardinal, Kuroda and Desiree was optimized. This protocol provides the base for the mass propagation and genetic engineering of these cultivars through *In vitro* techniques. Further investigation of the factors inducing microtuberization in micropropagated potato plantlets should be explored so that tissue culture technology can be applied for virus free stock and mass production.

Acknowledgement

Authors acknowledge the financial grant from Higher Education Commission of Pakistan.

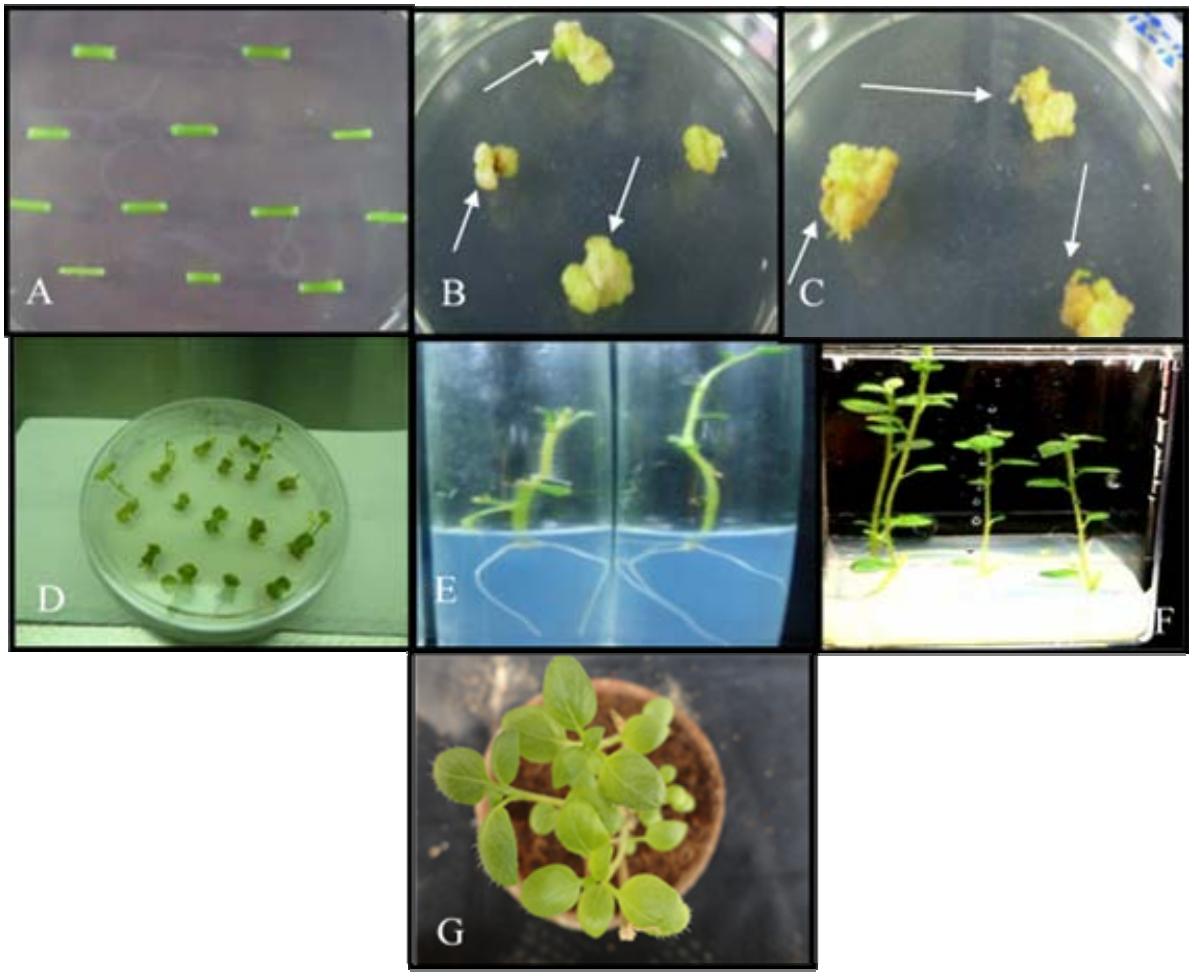

Fig. 3. Different regeneration stages for potato cultivars, A) Stem cuttings cultured on callus induction medium, B) Callus initiation, C) Bud formation (arrows), D) well developed shoots on shoot inducing medium, E) shoot cuttings transfer to root inducing medium; F) well-developed shoots transfer to magenta jars, G) complete plantlet of potato.

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(Received for publication 8 January 2015)