Protocol Optimization for \textit{In Vitro} Regeneration System in Frost Sensitive Potato Genotypes: As a Basis for Genetic Transformation Studies in \textit{Solanum tuberosum} L.

Siddra Ijaz\(^*\), Saba Idrees\(^1\) and Tariq Manzoor Khan\(^2\)

\(^1\)Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan
\(^2\)Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan

\textbf{Abstract}

For genetic transformation, an efficient \textit{in vitro} regeneration system in frost sensitive potato genotypes was established in this research study. Two frost sensitive genotypes of potato viz, Kuroda and FD-51-5 with good agronomic features but sensitive to frost were used. Six callus formation media were investigated for callus formation, in which three levels of 2, 4-D (3.5 mg/L, 4.5 mg/L and 5.5 mg/L) alone and in combination with one level of NAA (0.25 mg/L) were used. Of these, four callogenesis media viz, CM1, CM2, CM3 and CM4 were proved to be best for callus formation while genotype Kuroda gave best response. So these four CM and genotype kuroda was selected for \textit{in vitro} regeneration study in which five regeneration media (RM) were investigated. In these media three levels of BAP (2.75 mg/L, 3.75 mg/L, and 4.75 mg/L) and one level of NAA and kinetin (0.25 mg/L) were used in different combinations. For \textit{in vitro} regeneration, two age groups of calli viz, 28 days old and 35 days old were studies to check the effect of calli age \textit{in vitro} regeneration. These calli of genotype kuroda were induced on selected CM and then were put on these RM. Thereby results showed that twenty eight days old calli of genotype Kuroda from CM2 when shifted on RM4 gave maximum number of shoots.

\textbf{Keywords:} Kuroda; Frost; Low temperature tolerance; Tissue culture

\textbf{Introduction}

Potato (\textit{Solanum tuberosum} L.) is a food crop with great economic value. It is grown in temperate, tropical and sub-tropical regions [1]. This crop with multiple uses is attaining the status of staple crop. Potato is a staple food crop of forty countries around the world [2]. Potato is a tuberous crop plant which belongs to Solanaceae family [3]. Potato is a source of complex carbohydrates and antioxidants that provide energy and immunity to our body. The kind and amount of antioxidants may differ in different potato cultivars, though carotenoids and anthocyanin are predominant in this crop. It also provides a significant amount of vitamin C and potassium. The skin of medium size potato (5.3 ounce) contains 110 calories per serving.

This crop, because of high nutritional value, meets the increasing demand of food in the world. Potato crop is being cultivated in more than hundred countries and ranks fifth globally due to high production [4]. Potato is most important dicotyledonous tuber crop in the world [5]. Pakistan ranks at 7\textsuperscript{th} number worldwide, due to production of potato [6]. In Pakistan, potato is grown on an area of 104.5 thousand ha with 1684.7 thousand tons production per annum [7]. In Punjab, production of potato was 2767 thousand tons and yield was 24 thousand tons per hectare in 2007-2008. According to FAO report, potato production in 2010 was about 324 million tones in United Nations. Whereas in Pakistan its production was 2025 thousand tones and area under cultivation was 112 thousand hectare. However, during 2011-2012, potato production in Pakistan was 3392.5 thousand tons. This depicts that on an average, potato production in Pakistan is 2556.5 thousand tons from 2002-2012. In Pakistan current yield level of potato is 18 tons per hectare. In Pakistan three potato crops are achieved per year during autumn, summer and spring. Autumn crop is sown in October and harvested in January, summer crop is sown in January and harvested in May-June and spring crop is sown in April-May and harvested in August-September.

This crop has some environmental issues that severely restrict its production in terms of quality and quantity loss. Although plant growth and development are affected by both biotic as well as abiotic factors while abiotic stresses are the major cues that trigger various physiological and biochemical adaptations [8]. Biotic and abiotic factors adversely affect yield. Abiotic stress (temperature, chemical toxicity, drought, salinity and oxidative) are potential threats for potato crop [9]. Among these threats, frost is a major concern that is faced by potato crop. Frost causes different metabolic insults and yield deterioration in crop production [10].

At high latitudes frost damage is most important threat in potatoes [11]. Freezing temperatures restricted the length of the season suitable for the growth of potato crop. Frost damages potato crop when temperature becomes very low about -2\textdegree C. The lethal minimum temperature for the potato crop is 0\textdegree C. Intensity of frost injury depends on low temperature and duration of low temperature [12]. Ground frost was particularly severe during 2005-06 and 2007-08. Due to freezing, crystals form in tissues which lead to death of plant cell rapidly. Low temperature causes potato tuber injury and poor tuber germination.

During low temperature, a potato plant experiences three different stress conditions viz, dehydration, mechanical stress and osmotic stress i.e., due to the removal of water from vacuole. In dehydration stress, extracellular ice is formed while mechanical stress yields cell contraction as well as ice accumulation. Freezing injury of cell leads to ions leakage.
as well as turgidity loss. In addition to these panoramas, cell membrane disturbance, swelling of protoplasm and other plant organelles such as mitochondria and chloroplast are also the attributes of freezing injury [13]. Genetic engineering is becoming a useful technique and tissue culture is pre-requisite for this approach.

By considering frost as a major constraint of low production in potato crop, this study is designed that devoted exclusively to establish an efficient and expedite in vitro regeneration system in frost sensitive potato genotypes(s). Hence in this research study an in vitro regeneration system in selected genotypes of potato was established that would be used in genetic transformation studies in future. For developing frost tolerance through nonconventional ways, this would be a basis of genetic transformation strategies in potato.

Materials and Methods

Germlasm collection

Frost sensitive genotypes of potato viz, Kuroda and FD-51-5 were selected for this study. These genotypes were taken from Potato Research Institute, Sahiwal, Pakistan.

Media for callus induction

Six callus induction media containing different concentrations of plant growth regulators while basal MS salts and sucrose in common (Table 1) were investigated.

Explant sterilization and culturing on callogenesis media

Fresh and healthy potato tubers of studied genotypes were selected and surface sterilized by washing with water and dipped in laundry bleach containing 2 to 3 drops of tween 20, for 20 minutes. Then tubers were sprayed with 70% alcohol and cleaned with sterilized tissue paper. Thereafter under laminar flow hood tubers were sliced and then dipped into 70% ethanol for 1 minute following washing with ultrapure water. Thereafter these pieces were dipped into 25% bleach containing 2 to 3 drops of liquid soap for 20 minutes and then washed with ultrapure water. Subsequently in autoclaved distilled water these pieces were subjected to statistical analysis. The analysis of variance table revealed that significant variation was present among CM and between genotypes (Tables 3 and 4). However interaction variance table revealed that, significant variation was present among various treatments.

In vitro regeneration and root induction

For in vitro regeneration five regeneration media containing growth regulators (BAP, KIN, NAA) at different concentration along with Basal MS salt and sucrose in common were investigated (Table 1). But for the development of profuse rooting system, ½ MS media having no growth hormones was used. Proliferated calli of different ages viz, 28 days old and 35 days old were shifted to regeneration media, incubated for 6-8 hours in light/dark condition at 26 ± 1°C and were subcultured bi weekly. For root induction in vitro regenerated shoots were then shifted to ½ MS. Subsequent to rooting, plantlets were transferred into pots containing Belgium compost covered with polythene bags for acclimatization.

Collection and analysis of data

Data of callogenesis study were recorded in the form of rate of proliferation (see scale) and weight of calli for five weeks (Table 2). While in vitro regeneration data were collected as number of in vitro regenerated shoots/explants. Completely randomized design in factorial was used and Duncan’s multiple range tests was calculated among various treatments.

Results

Potato (Solanum tuberosum L.) is an economically important vegetable crop but it is prone to various biotic and abiotic threats. Among these, frost is most important factor that limits its yield. Hence, this research study was designed to establish in vitro regeneration system in selected frost sensitive potato genotypes. Because in vitro regeneration system (Tissue culture) is the pre-requisite or / basis for genetic engineering of crop plant.

Callogenesis

The response of callus formation was investigated on six different callogenesis media (CM). For callus induction study, three levels of 2, 4-D (3.5, 4.5 and 5.5 mg/L) and one level of NAA (0.25 mg/L) were used for both genotypes. Data for callus formation response of both genotypes were scored on the basis of callus proliferation rate and weight of Calli and subjected to statistical analysis. The analysis of variance table revealed that, significant variation was present among CM and between genotypes (Tables 3 and 4). However interaction table revealed that excellent proliferation rate as well as weight of calli were observed on CM1, CM2, CM3 and CM4 (Plate 1; Figures 1 and 2; Tables 5 and 6).

In vitro regeneration studies

On the basis of callogenesis study, genotype Kuroda and four callogenesis media (CM1, CM2, CM3 and CM4) were selected for in vitro regeneration study. Thus calli of selected genotype (Kuroda) were induced on selected CM and then were shifted on five regeneration media containing different growth regulators viz, KIN, BAP and NAA in different combination at different level. However, data were collected in the form of number of shoots per explant and statistically analyzed. Analysis of variance table revealed that significant variation was present between age of calli, among CM and among RM as well as their interaction (Table 7).

| Media composition | Callogenesis media (CM) | Regeneration media (RM) |
|-------------------|------------------------|------------------------|
|                   | CM1 | CM2 | CM3 | CM4 | CM5 | CM6 | RM1 | RM2 | RM3 | RM4 | RM5 |
| Nutrients Supplement | MSN | MSN | MSN | MSN | MSN | MSN | MSN | MSN | MSN | MSN | MSN |
| Sucrose (g/L)     | 30  | 30  | 30  | 30  | 30  | 30  | 30  | 30  | 30  | 30  | 30  |
| 2,4-D (mg/L)      | 3.5 | 4.5 | 5.5 | 3.5 | 4.5 | 5.5 | 3.5 | 4.5 | 5.5 | 3.5 | 4.5 |
| NAA (mg/L)        | 0   | 0   | 0   | 0.25| 0   | 0.25| 0   | 0   | 0   | 0   | 0.25|
| BAP (mg/L)        |     |     |     |     |     |     | 2.75| 3.75| 4.75| 4.75| 4.75|
| Kinetin (mg/L)    |     |     |     |     |     |     | 0.25| 0.25| 0   | 0   | 0   |
| Gellan gum powder (g/L) | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 | 2.66 |

MSN (MS salt=4.33 g/L, Myo-inositol=0.1 g/L, 500 µL vitamins (Nicotinic acid, Pyridoxine HCl, Thymine HCl, Glycine) pH=5.7-5.8

Table 1: Tissue culture media to be used in this study.
**Discussion**

In these study three levels of 2, 4-D (3.5 mg/L, 4.5 mg/L and 5.5 mg/L) and one level of NAA (0.25 mg/L) were studied for both genotypes (Kuroda and FD-51-5). Both genotypes showed different response on all CFM which depicts genotype based response. Kuroda genotypes (Kuroda and FD-51-5) were transferred into pots containing Belgium compost and then wrapped with polythene bag and were kept at 26 ± 1°C under 16/8 hr light-dark regime (Plate 8).

### Table 2: Scale to score callus proliferation.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F-value |
|---------------------|--------------------|---------------|--------------|---------|
| Week                | 4                  | 11.0667       | 2.767   | 5.50** |
| Genotype            | 1                  | 50.4169       | 50.4169     | 100.23** |
| CFM                 | 5                  | 31.3500       | 6.2700      | 12.46** |
| Genotype x CFM      | 5                  | 14.6833       | 2.9367      | 5.84** |
| Error               | 44                 | 22.1333       | 0.5030      |         |
| Total               | 59                 | 129.6500      |             |         |

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

### Table 3: Analysis of variance table for Rate of proliferation.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F-value |
|---------------------|--------------------|---------------|--------------|---------|
| Week                | 4                  | 0.29437       | 0.07359   | 5.55** |
| Genotype            | 1                  | 0.52547       | 0.52547     | 39.62** |
| CFM                 | 5                  | 0.28828       | 0.05766    | 4.35** |
| Genotype x CFM      | 5                  | 0.65158       | 0.13032    | 9.83** |
| Error               | 44                 | 0.58353       | 0.01326    |         |
| Total               | 59                 | 2.34323       |             |         |

** = Highly significant (P<0.01)

### Table 4: Analysis of variance (ANOVA) table for Weight of calli.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F-value |
|---------------------|--------------------|---------------|--------------|---------|
| Week                | 4                  | 10.667        | 2.767        | 5.50** |
| Genotype            | 1                  | 50.4169       | 50.4169      | 100.23** |
| CFM                 | 5                  | 31.3500       | 6.2700       | 12.46** |
| Genotype x CFM      | 5                  | 14.6833       | 2.9367       | 5.84** |
| Error               | 44                 | 22.1333       | 0.5030       |         |
| Total               | 59                 | 129.6500      |             |         |

** = Non-significant (P>0.05); * = Significant (P<0.05); ** = Highly significant (P<0.01)
using 2,4-D alone. Data were analyzed after five weeks of culture and the results showed that there was a wide range of callus initiation, callus health and rate of callus proliferation.

Among all growth regulators used 2,4-D was found to be the most effective growth regulator for potato callus induction when used alone. Castillo et al. [14] reported that auxin 2,4-D by itself or in combination with cytokinins has been widely used to enhance callus induction and maintenance. Moreover many researchers observed 2,4-D as the best auxin for callus induction as common as in monocot and even in dicot
Within different concentrations of 2, 4-D, when used alone highest degree of callus formation from tuber segment was recorded in MS medium. These results are agreement with our results. The necessity of cytokinin for shoot initiation is well documented by Ref. [15]. The success of callus induction and regeneration is dependent on genotype, the composition of the culture medium and the presence of appropriate combinations and concentration of hormones in the culture media. Many studies have been reported that in vitro callus induction was not only dependent on plant species but also on type of explants, light, temperature and explant age [5,21,22]. In potato, callus has been successfully induced from numerous explants, including leaf, stem segments including 1 or 2 node or without node (internode) and tuber [5,23,26]. According to the effect of hormones and their concentration on callus induction and regeneration [27] founded the highest percentage of callus induction (100%) was at 2.0 - 5.0 mg/L 2, 4-D alone.

It was investigated that 2, 4-D was best growth hormone among all other growth hormones (NAA, KIN and BAP) for rapid callus induction and for increasing rate of proliferation and weight of calli was not only dependent on plant species but also on type of explants, light, temperature and explant age [5,21,22]. In potato, callus has been successfully induced from numerous explants, including leaf, stem segments including 1 or 2 node or without node (internode) and tuber [5,23,26]. According to the effect of hormones and their concentration on callus induction and regeneration [27] founded the highest percentage of callus induction (100%) was at 2.0 - 5.0 mg/L 2, 4-D alone.

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Genotype Kuroda showed highest callus proliferation rate on media containing 2, 4-D alone. Similar results were observed by Shirin et al. [5] when they used 2, 4-D alone. They also studied growth regulators 2, 4-D, NAA alone and combination of NAA with BAP to check the

| CFM | RM | Age of calli | Mean |
|-----|----|--------------|------|
|     | 28 days | 35 days |
| 1   | 1.00 ± 0.00 fg | 1.00 ± 0.00 fg | 1.00 ± 0.00 F |
| 2   | 3.00 ± 0.58 ef | 6.00 ± 0.58 d | 4.50 ± 0.76 CD |
| 3   | 6.00 ± 0.58 d | 3.00 ± 0.58 ef | 4.50 ± 0.76 CD |
| 4   | 3.00 ± 0.58 ef | 12.00 ± 1.15 b | 7.50 ± 2.09 B |
| 5   | 0.10 ± 0.00 g | 0.10 ± 0.00 g | 0.10 ± 0.00 F |
| 1   | 1.00 ± 0.00 fg | 9.00 ± 0.58 c | 5.00 ± 1.81 C |
| 2   | 3.00 ± 0.58 ef | 4.00 ± 0.58 de | 3.50 ± 0.43 CD |
| 3   | 6.00 ± 0.58 d | 3.00 ± 0.58 ef | 4.50 ± 0.76 CD |
| 4   | 27.00 ± 1.75 a | 9.00 ± 0.58 c | 18.00 ± 4.11 A |
| 5   | 0.10 ± 0.00 g | 0.10 ± 0.00 g | 0.10 ± 0.00 F |
| 1   | 1.00 ± 0.00 fg | 1.00 ± 0.00 fg | 1.00 ± 0.00 F |
| 2   | 3.00 ± 0.58 ef | 2.00 ± 0.58 ef | 1.05 ± 0.50 F |
| 3   | 0.10 ± 0.00 g | 0.10 ± 0.00 g | 0.10 ± 0.00 F |
| 4   | 4.00 ± 0.00 fg | 6.00 ± 0.58 d | 3.05 ± 1.34 DE |
| 5   | 0.10 ± 0.00 g | 0.10 ± 0.00 g | 0.10 ± 0.00 F |
| 1   | 1.00 ± 0.00 fg | 1.00 ± 0.00 fg | 1.00 ± 0.00 F |
| 2   | 1.00 ± 0.00 fg | 2.00 ± 0.58 ef | 1.50 ± 0.34 EF |
| 3   | 0.10 ± 0.00 g | 1.67 ± 0.67 ef | 0.88 ± 0.46 F |
| 4   | 4.00 ± 0.00 fg | 0.10 ± 0.00 g | 0.10 ± 0.00 F |
| 5   | 0.10 ± 0.00 g | 0.10 ± 0.00 g | 0.10 ± 0.00 F |

Table 8: Age × CFM × RM interaction mean ± SE: Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

![Plate 8: Acclimatization of in vitro regenerated plantlets of cv. Kuroda, in pots containing Belgium compost.](image-url)
callus formation response. 2, 4-D was found best growth regulator for callus induction than other growth regulators. They used potato tuber for callus formation. They used 2, 4-D, NAA and BAP to test the callus induction response in potato (Solanum tuberosum L.). They observed massive amount of callus, when 2, 4-D was used alone. They concluded that 2, 4-D was best option for callus induction than all other growth regulators studied. Their results are similar with this study. Forrooghian et al. [29] studied three genotypes of potato viz, Santa, Agria and Savalan to observe the callus induction response. They used different concentrations of 2, 4-D and KIN for callus induction. They found 2, 4-D best growth regulator for callus induction. They concluded that higher concentration of 2, 4-D produced maximum callus. These results are similar with our results.

Kumar et al. [30] studied two potato genotypes (Kufri Chipsona 3 and MP- 97/644) for rapid callus induction. They found that 2, 4-D in combination with KIN produced best callus response in these two genotypes. They used BAP, KIN and AdSO, for shoot regeneration purpose. They observed that BAP in combination with AdSO produced best shoots. Our results have no similarity with these results. Khalafala et al. [27] used tuber as explant for callus formation in potato cultivar Almera. They found best callus response on media containing 2, 4-D alone. Their results agreed with our results. But for regeneration purpose, they used TDZ and BAP. They observed maximum regeneration on media containing TDZ hormone. In case of regeneration their results have no similarity with our results because in this study we observed maximum regeneration on media containing BAP alone. Ealeem et al. [23] used tuber explants for shoot regeneration. They used BAP and TDZ growth hormones for regeneration purpose. They observed maximum shoots on media containing TDZ alone. Their results are not in support of our results. Maximum number of shoots were observed on media supplemented with BAP alone with concentration 4.75 mg/L. our results have no similarity with the results obtained by Hussaini et al. [31] because they used combination of different growth hormones viz, BAP, GA, NAA and TDZ. They found best regeneration on media supplemented with combinations of BAP, GA, and TDZ.

BAP alone produced maximum number of shoots per explants. Our results are not matched with Ref. [5]. They used BAP, NAA, KIN growth regulators for shoot regeneration. They found that media containing KIN in combination with NAA was best for shoot regeneration. Laboney et al. [28] used two different growth regulators (BAP, GA) for shoot regeneration. They observed maximum number of shoots by using 1.0 mg/L BAP and 0.5 mg/L GA, in combination. Maximum number of shoots was observed by using 1.0 mg/L IAA and 1.0 mg/L GA, in combination. Their results are not in support of our results because in this study maximum number of shoots per explants was obtained by using BAP alone with 4.75 mg/L concentration. For root induction ½ MS was used.

Haque et al. [32] studied different explants and concentrations of 2,4-D and kinetin for callus formation. Then they observed weight of calli. They used explants which gave maximum calli when 1.0 mg/L 2,4-D and kinetin concentration was used. Similarly, different explants versus different concentrations of BAP/GA/IAA showed significant differences for shoot length and leaf number per plantlet and also for root length. However, interaction term confirmed node and node/internode explants produced better results in shoot length and number of leaves per plantlet when concentrations 1.0 mg/L BAP+0.1 mg/L GA, and 1.0 mg/L BAP+0.2 mg/L GA, 1.0 mg/L BAP+0.4 mg/L GA, respectively, were used. Shoot tip explants also produced better results in root length after 28 days plantlet when concentrations 1.0 mg/L IAA+0.25 mg/L GA, were used. These results have no similarity with our results.

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