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ROBUST REGENERATION PROTOCOL FOR THE *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION OF *SOLANUM TUBEROSUM*

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Abstract

Plant genetic transformation requires robust regeneration system. Plant growth regulators (PGRs) such as cytokinins (CKs) play a pivotal role in organogenesis; however, CKs are the most expensive PGRs. In the current study, an efficient yet economical protocol for regeneration of potato plant was developed. Stem inter-nodal and leaf explants were cultured on different regeneration media supplemented with varying concentration of different CKs such as kinetin and zeatin. Murashige-Skoog media added with zeatin (1, 1.5 mg/L) was designated as RZ_1, $RZ_{1.5}$, respectively or kinetin (1.5, 2 mg/L) was designated as $RK_{1.5}$ and RK_2, respectively, however, concentrations of other hormones such as NAA (1-Naphthaleneacetic acid) and GA_3 (Gibberellic acid A_3) were kept same. RZ_1 and $RZ_{1.5}$ gave significantly better results as compared to RK-type media in all aspects studied such as callus initiation, days to first shoot emergence, number of shoots per explants. RZ_1 medium was then selected as regeneration media for *Agrobacterium*-mediated transformation of potato plants with cyanobacterial *phosphoenol pyruvate carboxylase* gene, which provided multiple putative transformants on selection media. The transformants were further confirmed through PCR. The current protocol is found to be cost effective and efficient for the regeneration of *Solanum tuberosum* and can be successfully implied for the *Agrobacterium*-mediated transformation.

Key words: Tissue culture, Potato, Regeneration, *Agrobacterium* transformation, *PEPC*.

Introduction

Potato (*Solanum tuberosum*) is an important plant of the family *Solanaceae*, globally ranked fourth in cultivation (Newell *et al*., 1991). It is a rich source of carbohydrates, proteins, vitamins and fiber (Khalafalla *et al.,* 2010). Potato is said to be an economical crop as it gives more edible energy consuming lesser time and area (Badoni &Chauhan, 2010; Masarirambi *et al.,* 2012). However, it is vulnerable to many biotic (various viral, bacterial, fungal diseases and pest attacks) and abiotic stresses (salt, drought and high temperature etc.), which hamper its productivity and cause economic losses to agri-farmers throughout the world. (Ahmad *et al*., 2014) In addition to this, it also loses energy during photorespiration owing to its C_3-type of photosynthesis. Researchers are putting continuous efforts to enhance its tolerance against biotic/abiotic stresses but being a heterozygous plant, the process of varietal improvement via classical breeding methods is difficult and has limitations.

Here, genetic engineering technology can help to develop new germplasm with novel characters, to be utilized in breeding programs or to attain a status of new genetically modified cultivar. Many reports have been published which describe the genetic transformation of potato for its trait/quality improvement (Turhan, 2005; Tang *et al*., 2006; Sayari *et al*., 2005).

Genetic transformation of potato and many other crops demand a well-established and robust system for regeneration of new plant from a single cell using tissue culture. We have observed that researchers usually adopt a reported protocol and then seldom think to modify it, even, if it is costly. Many other researchers have produced studies on potato transformation in which higher concentration of plant growth regulators (cytokinins) was used which eventually became the protocol of choice (Ahmad *et al*., 2008; Ahmad *et al*., 2010; Tang *et al*., 2007; Kashani *et al*., 2012; Hoque, 2010). We hypothesized that there may be a possibility to regenerate potato plants on least possible concentration of cytokinin, which may be used as a protocol of choice for transformation studies.

Keeping this in view, the current study was designed to set up a regeneration system for potato plant that works efficiently yet being economical, by comparing the effect of two different cytokinins (zeatin and kinetin), which can be successfully used in the transformation protocol. We were successful to regenerate potato plants from leaf and stem explants on half concentration of the cytokinins previously used. Moreover, we were able to get multiple genetically modified shoots expressing *phosphoenol pyruvate carboxylase* gene from cyanobacteria. . Instead of a reporter gene, an important C4-pathway gene was used in plant expression vector for transformation. Thus successful regeneration of transformants will be the first step to engineer complete C4 photosynthesis pathway in potato in future.

Materials and Methods

Plant material: Sterile potato plants (cv. Desiree) were collected from Hazara Agriculture Research Centre, Abbottabad, Pakistan. The plants were propagated by nodal cutting on MS (Murashige & Skoog, 1962) media supplemented with 3% sucrose. All the further experiments were conducted on in-vitro propagated sterile plants.

Composition of different media and plant regeneration: For plant regeneration, leaf and stem internodes were used as explants. The explants were prepared and pre cultured on MS-medium supplemented with 2 mg/L of 2,4-D (callus induction media) for 4 days at 25ºC (under dark condition). After pre culturing, the explants were shifted to regeneration media. Explants were exposed to four different regeneration media (RZ_1, $RZ_{1.5}$, $RK_{1.5}$ and RK_2) each with a varying concentration of cytokinin (Table 1). The plants were cultured at 25ºC in the growth chamber with regular shifting to fresh media after every 21 days. The effect of media on plant regeneration (callus induction, shoot initiation, shoots per explants etc.) was recorded. Shoots that successfully regenerated were shifted to rooting media (MS media).

Construction of plant expression vector: Plant expression vector pCyn*PEPC* was constructed by amplifying the coding region using cyanobacterial (*Synechocystis* sp. PCC 6803) DNA. The resultant amplified gene was sequenced and compared with reported sequence. Then the coding region was loaded downstream of CaMV 35S promoter region and upstream of 35S terminator region. This cassette was finally loaded on pCAMBIA 2300 having kanamycin as a plant selection marker (Fig. 1). The plant expression vector was transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method (Holsters *et al*., 1978).

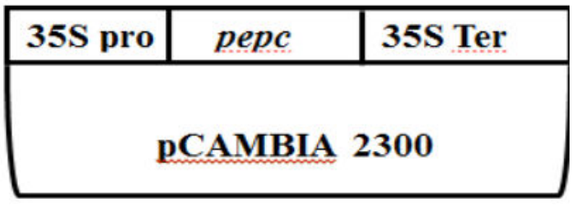


Fig. 1. Schematic representation of plant expression vector diagram used for transformation of potato plants. 35S Pro. Cauli flower mosaic virus promoter, *PEPC*: coding region of *phosphoenolpyruvate carboxylase* gene of PCC 6803, 35S Ter.: Terminator region

***Agrobacterium* mediated plant transformation of potato plant:** For plant transformation, *Agrobacterium* with *PEPC* gene was cultured in 5 mL of YEP (Yeast extract peptone) medium (supplemented with 100 mg/L kanamycin) for 2 days at 28˚C. The transformation protocol described by Ahmad *et al*., 2008 was followed. Briefly, leaf and inter-nodal explants were prepared and pre cultured on MS media supplemented with 2 mg/L 2, 4-D (pre culture medium) for 2 days in dark. These explants were inoculated with *Agrobacterium* for 10 min, in liquid MS medium and then wiped on sterile tissue paper and put on pre-culture medium for another two days in dark. The explants were then shifted to regeneration medium (RZ_1) supplemented with 350 mg/L cefotaxime and 100 mg/L kanamycin (for the selection of transformants). The explants were transferred to fresh medium after every third week. The shoots that successfully regenerated were shifted to rooting media (MS media with 350 mg/L cefotaxime and 100 mg/L kanamycin). Plantlets that successfully rooted on selection media were further screened for the integration of T-DNA region using genomic DNA PCR.

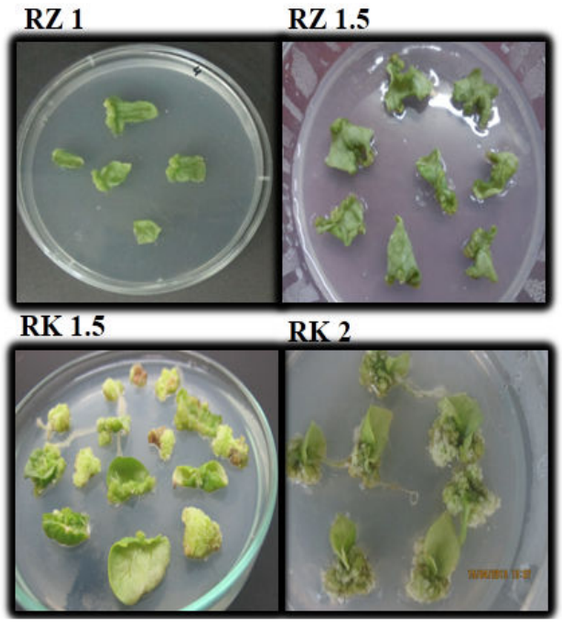
PCR mediated screening of putative transformants: Genomic DNA was extracted from the transformants using the CTAB method defined by Doyle and Doyle (1987). The PCR reaction was carried out with the purified DNA using the primers specific for *NPTII* (neomycin phosphotransferase) gene. The amplification reactions was carried out at 94˚C for 5 min (1 cycle), followed by 30 cycles (94˚C 30 s, 62˚C 30 s and 72˚C 1 min.) and lastly an extension cycle of 7 min at 72˚C. The PCR product was gel electrophoresed using 1% agarose gel stained with ethidium bromide, and then visualized under UV.

Statistical analysis: The results were analyzed statistically. Variation within the replications was determined by standard deviation, using Microsoft excel. Significant difference between treatments (media) was calculated using Student's *T-test* at 0.05 level of significance.

Results

Effect of 2, 4-D in combination with different cytokinins on callus induction: To induce callus, leaf and intermodal explants were cultured on 2, 4-D-supplemented MS medium for 4 days in dark. After 4 days of culture, swelling on the cut surfaces of the explants was observed, however, a visible clump of callus was not observed. After 4 days of culture on 2, 4-D supplemented MS-medium, explants were shifted to different media (Table 1) meant to induce regeneration. It was observed that callus formation initiated earlier on explants cultured on $RZ_{1.5}$ medium whereas explants on RK_2 took the maximum time for callus initiation. Explants on both concentrations of zeatin formed compact callus whereas the explants on kinetin media mostly yielded friable callus (Fig. 2a). Explants on $RZ_{1.5}$ took 5 days for callus initiation, whereas callus initiated on explants of RZ_1, $RK_{1.5}$ and RK_2 in 9, 8 and 10 days, respectively. The statistical analysis depicted that $RZ_{1.5}$ gave significantly earlier callus induction as compared to other media (Fig. 2b).

Effect of different concentrations of cytokinins on shoot organogenesis: To evaluate the effect of different cytokinins on shoot organogenesis, explants were cultured for total 10 weeks on already described media i.e., RZ_1, $RZ_{1.5}$, $RK_{1.5}$ and RK_2. Contrary to days to callus induction results, RZ_1 induced shoots in 28 days. However, other media such as $RZ_{1.5}$, $RK_{1.5}$ and RK_2 took 30, 34 and 33 days, respectively. Statistically the early shoot initiation on RZ_1 was evidenced significant at $\alpha = 0.05$(Fig. 3). We did observe that RZ_1 media produced thin shoots as compared to other media; however, all those shoots developed roots successfully. Interestingly, RZ_1 media depicted on average 8 shoots per explants, while $RZ_{1.5}$, $RK_{1.5}$ and RK_2 produced 3 and 2 shoots per explants (Fig. 4). Statistically RZ_1 values were significantly different ($\alpha = 0.05$) from the results obtained from other media employed for regeneration to evaluate regeneration capabilities.



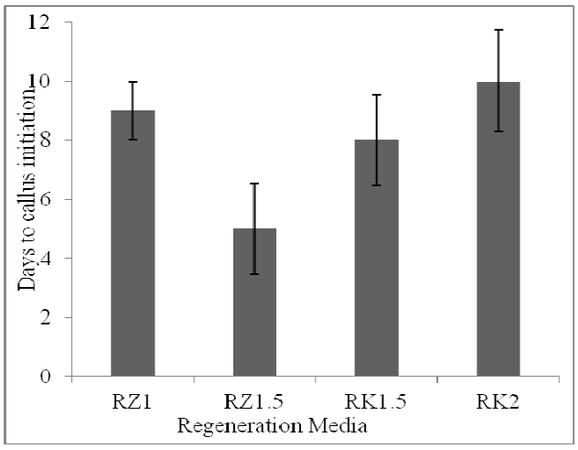
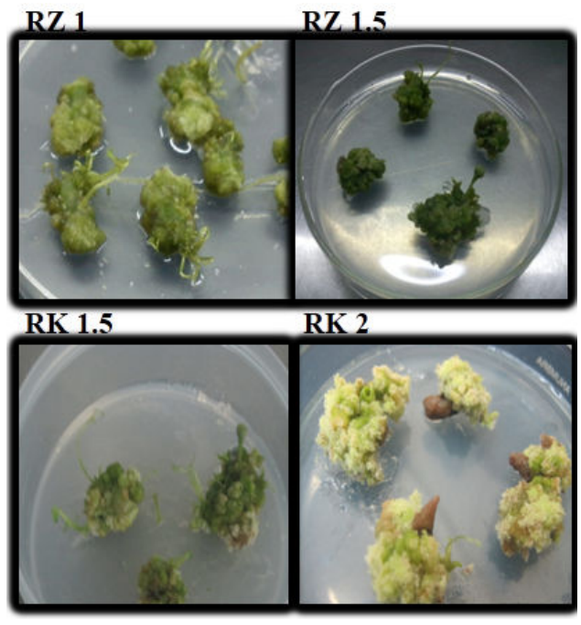


Fig. 2. Effect of different concentrations of zeatin and kinetin on callus initiation. a) Representative photograph of explants showing callus. b) The bars depict the days to callus initiation on different media. The values are the means ± standard deviation. * shows significantly varying treatment at ($p<0.05$) according to Student's t-test



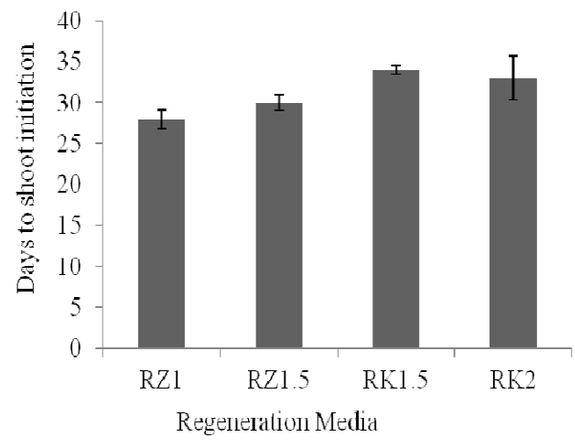


Fig. 3. Effect of different concentrations of zeatin and kinetin on shoot initiation. a) Representative photograph showing shoot emergence from explants. b) Different regeneration media plotted against the days taken by explants for shoot initiation on that media. Data are expressed as the mean ± standard deviation. * shows significantly varying treatment at ($p<0.05$) according to Student's t-test.

Efficiency of different cytokinins to regenerate potato shoots: *In vitro* plant regeneration is affected by a number of factors. We observed that all the explants cultured on regeneration media, did not successfully yield shoots. Although the explants put on $RK_{1.5}$ and RK_2 successfully formed the callus but the callus being friable, rarely initiated the shoot formation. There was only 44% regeneration from the explants cultured on RK_2. The maximum percent regeneration was observed from RZ_1 medium i.e., 92% of explants on RZ_1 medium regenerated successfully, while $RZ_{1.5}$ and $RK_{1.5}$ provided 86 and 46% regeneration, respectively. Statistical analysis verified that RZ_1 and $RZ_{1.5}$ provided significantly higher regeneration as compared to other media (Fig. 5).

Transformation of *PEPC* gene into potato plants using tailored regeneration media: After successfully developing and evaluating a simple yet economical regeneration protocol which utilizes half concentration of zeatin as compared to previously used concentrations (Table 2), we transformed *PEPC* gene into potato plants using RZ_1 medium supplemented with kanamycin as a selection marker. Three rounds of transformation experiment were conducted. Quite a good number of shoots were regenerated on selection marker containing regeneration medium after 30-35 days. The putatively transformed shoots were cut from the explants and transferred to rooting medium. PCR based further screening was performed to validate the transformation and integration of expression cassette into potato genome by using *NPTII* gene specific primers. Multiple shoots showed presence of *NPTII* gene. A representative figure of PCR is shown (Fig. 6).

Table 1. Various combinations of hormones and their respective concentrations used for regeneration of potato supplemented in MS-medium.

Media	Hormone concentrations
RZ_1	0.01mg/L NAA+0.1mg/L GA_3+1mg/L Z
$RZ_{1.5}$	0.01mg/L NAA 0.1mg/L GA_3+1.5mg/L Z
$RK_{1.5}$	0.01mg/L NAA+0.1mg/L GA_3+1.5mg/L Kn
RK_2	0.01mg/L NAA+0.1mg/L GA_3+2mg/L Kn

Table 2. Representative studies for potato regeneration, using comparatively higher concentrations of different cytokinins.

No.	Cytokinin used	Concentration (mg/L)	Reference
1.	Zeatin	2	Ahmad *et al*., 2008; Ahmad *et al*., 2010; Tang *et al*., 2007; Dhital *et al*., 2011
2.	Zeatin	2.5	Zel & Medved, 1999
3.	Zeatin	4	Kashani *et al*., 2012
4.	Kinetin	2.0	Hoque, 2010

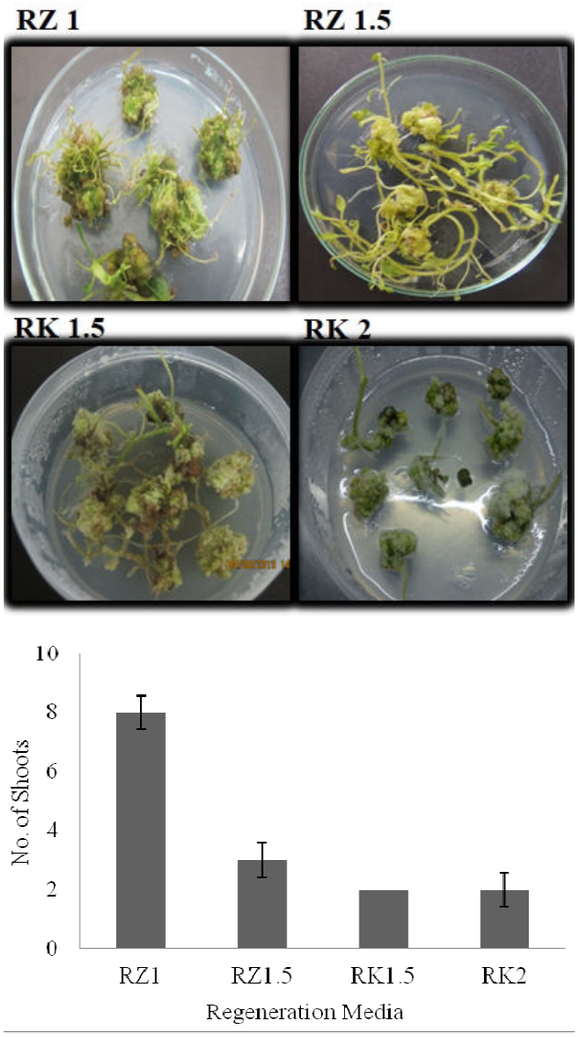


Fig. 4. Effect of zeatin and kinetin on multiple shoot organogenesis. a) Representative photograph depicting multiple shoots emerging from explants. b) The bars showing the average No. of shoots per explants cultured on different concentrations of zeatin and kinetin. Data are expressed as the mean ± standard deviation. * shows significantly varying treatment at ($p<0.05$) according to Student's t-test.

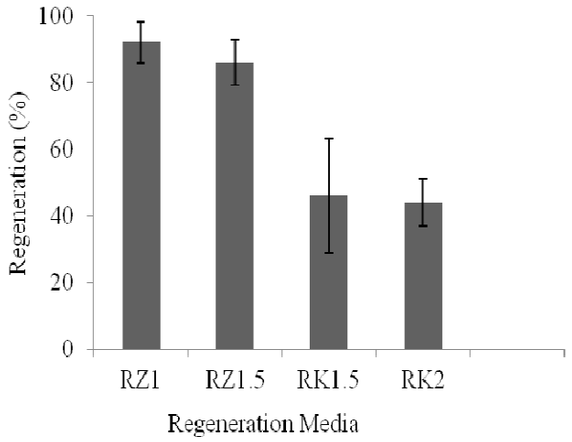


Fig. 5. Percentage of explants which regenerated shoots. Explants were cultured on regeneration media for total 10 weeks. Explants from which shootsiniated successfully were calculated and percentage of successful regenerants was calculated. Data are expressed as the mean ± SD. * shows significantly varying treatment at ($p<0.05$) according to Student's t-test.

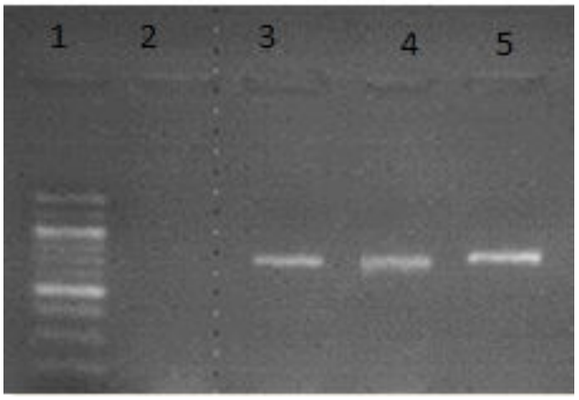


Fig. 6. Transformation of potato plants using cyanobacterial *PEPC* gene by using RZ_1-regeneration media optimized in this study. Representative photograph of PCR analysis of regenerated shoots after transformation by using *NPTII* gene specific primers. SM: size marker, NC: negative control, T1, T2: independent transgenic lines showing bands corresponding to *NPTII* gene, PC: positive control.

Discussion

Both auxin and cytokinin play a vital role in cell division. It is also believed that cytokinins have different role in cell division than auxins (Sakakibara, 2006; Su *et al*., 2011). Auxin (especially 2, 4-D) has been predominantly used for callus induction in variety of experiments and for diverse plant species (Noor *et al*., 2009; Malik *et al*., 2003). Auxins are absorbed by the explants within hours (Woodward, 2005) which explants may utilize to promote cell division later on. To expedite and maintain cell division cytokinins are used in tissue culture (Sakakibara, 2006, John *et al*., 2014). The results in our study showed that zeatin has more synergistic effects along with 2, 4-D to initiate and promote cell division and to initiate differentiation. Moreover, zeatin based medium produced mostly compact callus as

compared to kinetin based media, which leads to organogenesis. This depicts that zeatin is more potent cytokinin for shoot organogenesis supporting the work carried out by Molla *et al*., 2011.

Cytokinins are known to play the key role in plant regeneration. When added to the culture media, cytokinins lead to shoot formation but not all cytokinins are supposed to function in a similar manner. Some cytokinins in a very small quantity enhance organogenesis. On contrary, some cytokinins, even in increased concentration may not have any profound effect on plant regeneration (Arshad *et al*., 2012; Haberer & Kieber, 2002). Among the cytokinins used in the current study, shoots originated earliest from the media having zeatin. Early shoot generation using zeatin as the cytokinin in the culture media has been reported by (Kashani *et al*., 2012). According to (Kashani *et al*., 2012), use of zeatin in the culture media reduces the duration of callus phase resulting in early shoot formation. On contrary to zeatin, explants on media supplemented with kinetin took more time for shoot regeneration. The results of the study were in accordance with the findings of Badoni & Chauhan, 2009. Inefficiency of kinetin for shoot development in potato plant has been reported by Badoni & Chauhan, 2009. According to the study carried out by them, increasing the concentration of kinetin in regeneration media, resulted in delayed shoot development, consequently reducing the shoot height and weight. The results of our study supported this fact.

The use of zeatin for potato regeneration has been reported (Zel & Medved 1999; Ahmad *et al*., 2008; Kashani *et al*., 2012) but they used zeatin (2-mg/L) in high concentration. So, the current study gave a cost effective complement by reducing the concentration of cytokinin in the medium. The reason why zeatin 1mg/L gave more number of regenerated shoots has been explained with the finding of Vikram *et al*., 2012 which shows that adding the auxin along with zeatin (low concentration), produced more shoots per explants than that of higher concentration of zeatin. The media was further used for the regeneration of transformants. The transformants successfully regenerated on the media having kanamycin as a selection marker and showed stable integration via PCR.

Conclusions

To conclude, zeatin proved to be more potent cytokinin to induce shoot regeneration in potato plants as compared to kinetin. The lower concentration of zeatin produced more number of regenerants per explants, albeit little weak shoots than those regenerated on higher zeatin containing medium. The optimized regeneration protocol was equally good for genetic transformation studies as well, in which we successfully obtained multiple PCR positive lines expressing *PEPC* gene, which is a first step to engineer complete C_4 pathway into potato plants.

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