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EXPLORING THE AMENABILITY OF ONIONS TO *AGROBACTERIUM* MEDIATED TRANSFORMATION

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ABSTRACT

The onions exhibit recalcitrant nature when subjected to genetic modifications. The study was conducted to optimize various factors affecting *Agrobacterium* mediated transformation in onion. Factors such as genotype dependency, type of explants and response of growth regulators were investigated. *Agrobacterium* strain LBA4404 harboring pBIN19 binary vector was used to infect different explants of two cultivars (Kral and Sampiyon). The T-DNA region of vector contained *uidA* gene (interrupted by an intronic sequence) to screen primary transgenic shoots at earlier stage and *nptII* gene as plant selectable marker. Results exhibited the genotype and explant dependency towards genetic modification of both cultivars. Based on the rate of callus induction, regeneration response and GUS histochemical assays, cultivar "Kral" was found better compared to "Sampiyon". The best callus induction was observed on MS medium supplemented with 1:10 ratio of 2,4-D and BAP respectively. Out of 355 primary transformants, 87 primary transformants were recorded as positive when subjected to PCR assays; 51 belonging to Kral showing tendency of genotype to genetic improvement. Results showed that highest number of putative transgenic plants was contributed by mature embryos followed by seeds and basal plates explants. Onion seed as explant has been used successfully for first time as no evidence in earlier literature was found. The optimization of these factors in *Agrobacterium tumefaciens*-mediated transformation will provide a gateway to introduce any desired trait in onion.

Keywords: Genetic transformation, onion, regeneration, GUS analysis, transgenes.

INTRODUCTION

From the ancient times, the conventional plant breeding has been a great tool to improve the crops from economical point of view. The problem with the conventional plant breeding methodologies is their unpredictability as it may lead to several years of careful work of breeding to develop a plant with desirable characteristics (Danida, 2002). Genetic transformation technologies have enabled the scientists to introduce any trait of economic importance in crops by breaking the barriers across species. These technologies transcend traditional plant breeding methodologies by allowing the rapid and predictable gene transfer across the species boundaries (Pimental *et al.* 1989). Potential benefits include herbicide resistance, insecticide resistance and enhanced nutritional profile of crops (Pimental *et al.* 1989).

Agrobacterium mediated transformation is one of the most reliable methods of genetic transformation in plants. It is being used for genetic transformation of many crops. The protocol of *Agrobacterium* meditated transformation is relatively simple, straightforward, economical, and the most important; it results in the insertion of single transgene (Hansen and Wright, 1999). This transformation system has been long established in dicotyledonous plants (Fraley *et al.* 1986). Therefore, most monocotyledonous plant species being unresponsive to *Agrobacterium*-mediated transformation have been subjected to direct gene transfer techniques in monocots species. But these direct gene transfer techniques are not preferred much by scientists mainly due to insertion of multiple copies of transgene (Christou, 1997; Christou, 1995).

Onion belongs to monocotyledonous group poses less response to genetic transformation due to large genome size (Arumuganathan and Earle, 1991) and the main factors which decrease the efficiency *of in vitro* onion regeneration are bulblet formation, tissue virtification and plantlets dormancy (Eady *et al.* 2003). Previously this group was assumed as a difficult crop for genetic transformation due to lack of an optimized and reproducible protocol (Eady, 1995c). An improved strategy directed to strengthen the *Agrobacterium* mediated transformation system is required to overcome barriers to transform monocots genetically.

Development of an efficient and reproducible plant transformation and regeneration protocol is a prerequisite for genetic transformation studies of plants (Sivanesan *et al.* 2015). It provides a gateway to introduce any desired trait in targeted plant. Successful plant transformation requires a proper DNA delivery system, a plant regeneration system, and a selection system to recognize the transgenic cells. For onion, the

characterization of these transformation aspects is still lacking and needs to be optimized for an efficient genetic transformation (Eady and Lister, 1998b). Most of the earlier studies on *Agrobacterium tumefaciens* mediated transformation in onions report the use of immature embryos as explant that is laborious, time specific and contamination associated step. The present study was conducted to explore the possibility of use of different explants and growth regulators for genetic manipulations of onions.

MATERIALS AND METHODS

The seeds of good yielding onion cultivars (but susceptible to pests and diseases) were provided by MTN seeds, Bandirma, Turkey. For the present study, Kral and Sampiyon were selected based on their *in vitro* germination response. The cultures were incubated at 24±2°C with 16 hour lights (35µmpl photons m-2 s-1) photoperiod cool white fluorescent tubes.

Seed Sterilization: Forty eight seeds of Kral and Sampiyon cultivars were surface sterilized by immersing in 100 ml sterilized water containing two drops of Tween-20, for 15-20 minutes with continuous agitation. Later on, seeds were further surface disinfested by immersing in 70% ethanol for 2 min and 2% H_2O_2 for 15-20 min and then rinsed in sterilized deionized water for 4-5 times. The seeds were dried on autoclaved filter paper and cultured in triplicate on basal MS medium containing Murashige and Skoog mineral salts, 3% sucrose and 7 g/l agar and incubated in growth chamber at ambient temperature and light conditions to obtain explants (shoot and root tip, basal plate, leaf blade) for genetic transformation experiments. The sterilized seeds were also placed on moist filter paper in petri plates to obtain mature germinating embryos (2-3 days) as explants.

Genetic Transformation: *Agrobacterium* strain LBA4404 harboring pBIN19 binary vector containing *uidA* gene (βeta-glucuronidase) under the control of 35S promoter was used (Figure-1). *uidA* gene was interrupted by an intronic sequence to induce expression only from eukaryotic cells. The glycerol stock was streaked on Lauria Broth (LB) plates containing kanamycin and rifampicin each at concentration of 50 mg/l. One colony from plate was inoculated in LB broth supplemented with appropriate concentrations of antibiotics. The bacterial culture was incubated in thermo-shaker at 28°C for overnight.

Explants were subjected to *Agrobacterium* mediated transformation, with no prior pre-culture. The explants were inoculated with *Agrobacterium* suspension (O.D 0.6) for 45 minutes with gentle shaking in liquid medium without antibiotic followed by incubation on co-cultivation medium for three days. Co-cultivation medium contained MS salts including vitamins, 3% sucrose, 7-8 g/l agar and 100 µM acetosyringone. The pH of medium was adjusted to 5.8.

Following co-cultivation with *Agrobacterium*, the explants were washed with antibiotic and incubated on regeneration selection medium (RSM).Three different media (Table-1) were tested to optimize the conditions for in-vitro onion regeneration. Each RSM contained Duocid, a broad spectrum antibiotic with ingredients of ampicillin + Sulbactam Sodium to suppress the growth of *Agrobacterium*. With well-developed calli and micro-shoots were transferred to shoot/root medium (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/l GA3, 500 mg/l Duocid and 100 mg/l kanamycin). When plantlets reached to a certain size in shoot elongation media with rooting as well, putative transgenic plants were shifted to pots containing 3:1 mixture of organic matter and perlite in green house.

GUS Histochemical Assay: The expression of *uidA* gene was studied through histochemical X-Gluc assay. GUS solution was prepared containing 10mg/L X-Gluc, 10mM EDTA, 100mM NaH_2PO_4, 0.1% Triton X-100 and 50%methanol. The pH was adjusted to 8.0. The solution was protected from light. The regenerated shoots/explants transformed were dipped in X-Gluc solution in an eppendorf and kept at 37^0C for one hour to overnight. The GUS activity was stopped by washing with 70% ethanol until de-staining was complete.

PCR Analysis: Total 355 primary transformants were recovered from *in vitro* regeneration experiments and were subjected to PCR analysis to confirm the presence of introduced gene. DNA extraction was done by CTAB method according to the protocol given by (Murray *et al.* 1980). DNA concentrations were checked through Nanodrop spectrophotometer.

PCR analysis was done to confirm presence of transgene in plants. For 1x PCR reagents, 12.5 µl 2x-mix, 0.5 µl (10mM) of each forward and reverse primer, 5 µl (5 ng/µl) template DNA and 6.5 µl ddH2O were mixed to make 25 µl total reaction volume. PCR program for *nptII gene* was followed as initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 58°C for 45 seconds, extension at 72°C for 1 minute with 34 rounds of amplification and final extension at 72°C for 7 minutes. PCR program for *uidA* gene was same except annealing temperature of 58°C. The plasmid DNA was used as positive controls in both PCRs (for *nptII* and *uidA* genes). For wild control, non-transformed DNA "B17-50B" was used. The putative transgenic plants were subjected to PCR to check the absence of chromosomal virulence gene (Chv-A) by using gene specific primer. *Agrobacterium* strains LBA4404 harboring PTF-101.1 plasmid was used as positive control. PCR products were resolved using 1.5% agarose gel.

After compiling all data, the effect of genotype and growth regulators on transformation efficiency was

evaluated. The transformation efficiency of each explant was calculated by dividing number of GUS and PCR positive plants to total number of explants used.

RESULTS

The onion varieties "Sampiyon" and "Kral", with average germination percentages of 93 and 78% respectively were selected for the proposed study (data not shown). The varying response of each explant was observed on RSM. On RSM-1 (Table-2), regeneration was observed in mature embryos and seeds explants of both varieties. Basal plates of both varieties showed callus formation and regeneration. Shoot and root tips did not show any response on RSM-1, while dark green calli were observed in leaf blades of both varieties. On RSM-2 (Table-3), the explants showed higher frequency of callus induction compared to RSM-1. Mature embryos, basal plates and root tip showed callus induction. Direct regeneration was observed in seeds of both varieties. Shoot tips of Kral and Sampiyon did not show any response. Dark green calli were observed in leaf blades. On RSM-3 (Table-4), the frequency of callus induction was very less. Regeneration was observed in basal plates, seeds and mature embryos. Shoot tips, root tips and leaf blades did not show any response.

Well-developed onion micro-shoots (obtained from regeneration medium) were transferred to shooting and rooting medium to develop in vitro transgenic plants. No any problem of rooting in primary transformants was recorded. Shoot elongation media resulted in robust rooting in both cultivars. The putative transgenic plants were transferred to soil for acclimatization.

The regenerated shoots subjected to GUS assay turned blue due to GUS activity ensuring transformed cells. Figure-2 shows various regeneration response and GUS activity. The explants of both varieties showed GUS staining except root tips. The GUS expression in root tips was very recorded low.

In order to confirm presence of introduced genes (*nptII* and *uidA* genes) in primary transformants, PCR analysis was done of total 355 plant samples. 87 plant samples gave positive PCR results confirming that presence of transgene in plant genome. Transgenic plants showed 450 bp band size (for *nptII* gene) and 362 bp band size (for *uidA* gene) on agarose gel (Figure-3 and 4). Moreover, PCR was conducted to check the *Agrobacterium* contamination in putative transgenic plants. The results exhibited the absence of 890 bp band size which ensured the absence of any agrobacterium contamination in primary transformants (Figure 5).

In the total PCR positive plants obtained, highest frequency of transgenic plants was contributed by mature embryos followed by seeds and basal plates. None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades in the transformation experiments performed.

Basal plates showed good transformation efficiencies on RSM-1 and RSM-2. Mean transformation frequency for basal plates on RSM-1 was 1.1% for Sampiyon and 8% for Kral (Figure-6&7). Mean transformation frequency for basal plates on RSM-2 was 4.4% for Sampiyon and 7% for Kral. On RSM-3, transformation ability of basal plates was zero in Kral and in case of Sampiyon; the mean transformation frequency was 3.3%. In the case of genotype effect, basal plates of both varieties gave good transformation efficiencies on RSM-1 and RSM-2, however, in case of RSM-3, Sampiyon performed well. On other two RSM, Kral was observed performing well overall (Figure-6&7).

Mature embryos showed good regeneration on RSM-1 and RSM-2. Mean transformation frequency for mature embryos on RSM-1 was observed 9% for Sampiyon and 17% for Kral. Mean transformation frequency for mature embryos on RSM-2 was 4.4% for both varieties. In case of genotype differences on media, Kral was better on RSM-1 with 17% transformation efficiency. On RSM-3, the transformation efficiency was zero (Tables 2-4).

Seeds explant of both cultivars performed well on all regeneration media. However, good transformation and regeneration efficiencies were observed on RSM-1 and RSM-2. Mean transformation frequency for seeds on RSM-1 was observed 10% for Sampiyon and 12% for Kral. Mean transformation frequency for seeds on RSM-2 was 7% for Sampiyon and 8% for Kral. Kral performed well as compared to Sampiyon on both RSM media. On RSM-3, transformation efficiencies were not as good as on RSM-1 and RSM-2 (Tables 2-4). Shoot tips, root tips and leaf blades did not show any response on regeneration media. None of positive transgenic plant was obtained from them.

For each medium, genotype efficiency for transformation was different. However, in present study, Kral performed well overall. In the total of 87 onion transgenic plants, 51 contributed by Kral and 36 obtained from Sampiyon. The highest transformation frequency obtained was 17% of mature embryos explants followed by 12% by seeds and 8% by basal plates.

Table 1. Composition of different RSM used for onion *in vitro* regeneration.

Reagents	RSM-1	RSM-2	RSM-3
MS salts	4.4 g/l	4.4 g/l	4.4 g/l
Sucrose	30g/l	30g/l	30g/l
pH	5.8	5.8	5.8
Agar	8 g/l	8 g/l	8 g/l
BAP	2mg/l	0.05 mg/l	2mg/l
NAA	0.2 mg/l	--	2 mg/l
2,4-D	--	(0.5 mg/l)	--
Kanamycin	100 mg/l	100 mg/l	100 mg/l

Table 2. Response of Explants on RSM-1 and Shoot/Root Media used in the study.

Variety	Explants used	Total # of Explants	Callus induction& Proliferation %	Regeneration %	No. of plants on Shoot/Root Medium	Plant transferred to soil	PCR positive Plants	Transformation Efficiency %
Sampiyon	Basal Plates	90	24.4(0)	16.22	13	9	1	1.1
	Mature Embryos	90	0	93.3	84	49	8	9
	Seeds	90	0	45.6	41	25	9	10
	Shoot Tips	90	18.9*(0)	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0
	Leaf Blade	90	77.8*(0)	0	0	0	0	0
Kral	Basal Plates	90	8.9(0)	21	17	12	7	8
	Mature Embryos	90	0	60	54	22	15	17
	Seeds	90	0	47.8	43	30	11	12
	Shoot Tips	90	20*(0)	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0
	Leaf Blade	90	44.4*(0)	0	0	0	0	0

The * symbol is indicating Dark green callus.

Table 3. Response of explants on RSM-2 and Shoot/Root Media.

Variety	Explants used	Total # of Explants	Callus induction& Proliferation %	Direct Regeneration %	No. of plants Shoot/Root M	Transferred to soil	PCR positive	Transformation Efficiency %
Sampiyon	Basal Plates	90	67.8(0)	25	21	18	4	4.4
	Mature Embryos	90	35.6(0)	42.2	35	18	4	4.4
	Seeds	90	0	90	81	35	6	7
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	17.8(0)	0	0	0	0	0
	Leaf Blade	90	32.2*(0)	0	0	0	0	0
Kral	Basal Plates	90	68.9(0)	27.8	25	12	6	7
	Mature Embryos	90	40.6(0)	55.1	48	23	4	4.4
	Seeds	90	0	86.7	78	23	7	8
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	13.3(0)	0	0	0	0	0
	Leaf Blade	90	52.2*(0)	0	0	0	0	0

The * symbol is indicating Dark green callus.

Table 4. Response of explants on RSM-3 and Shoot/Root Media.

Variety	Explants used	Total # of Explants	Callus induction & proliferation %	Direct Regeneration %	Shoot/Root medium	Transferred to soil	PCR positive Plants	Transformation Efficiency %
Sampiyon	Basal Plates	90	0	90	81	10	3	3.3
	Mature Embryos	90	0	94.4	85	4	0	0
	Seeds	90	0	87.8	79	18	1	1.1
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	7.7(0)	0	0	0	0	0
	Leaf Blade	90	0	0	0	0	0	0
Kral	Basal Plates	90	10(0)	82.2	73	17	0	0
	Mature Embryos	90	0	80	72	6	0	0
	Seeds	90	0	87.8	79	24	1	1.1
	Shoot Tips	90	0	0	0	0	0	0
	Root Tips	90	0	0	0	0	0	0

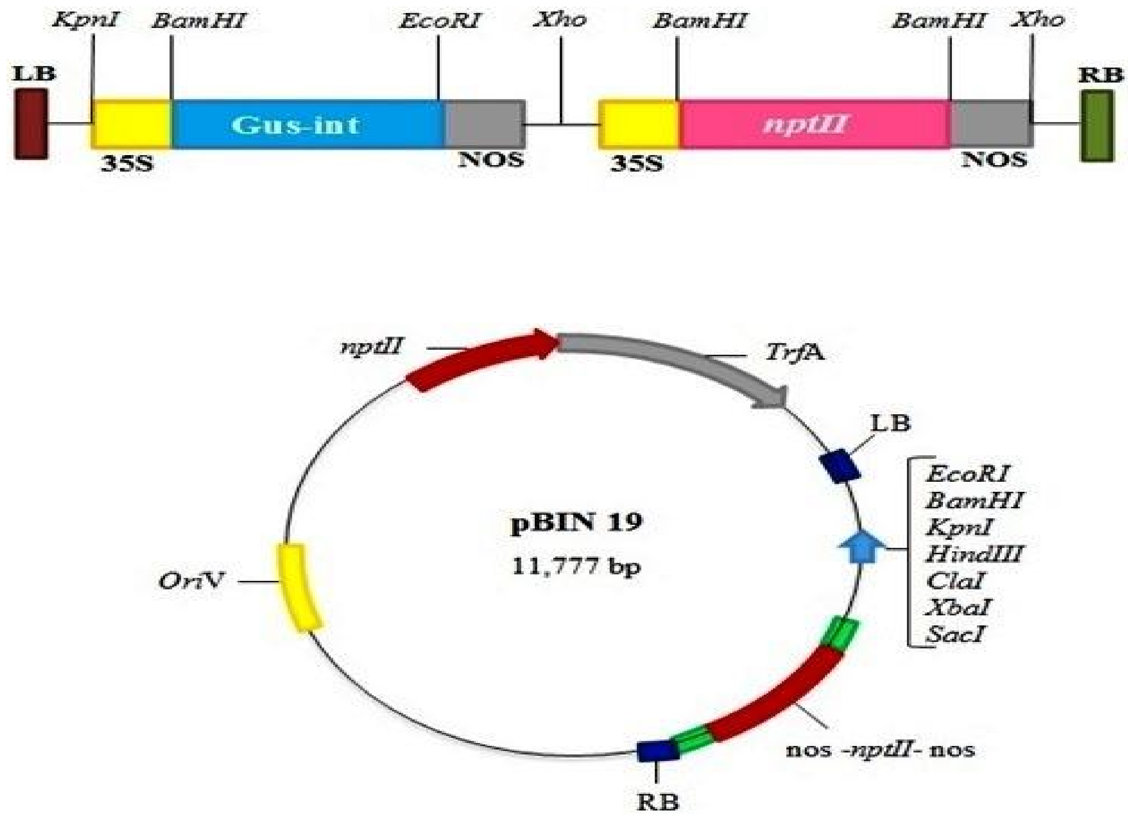
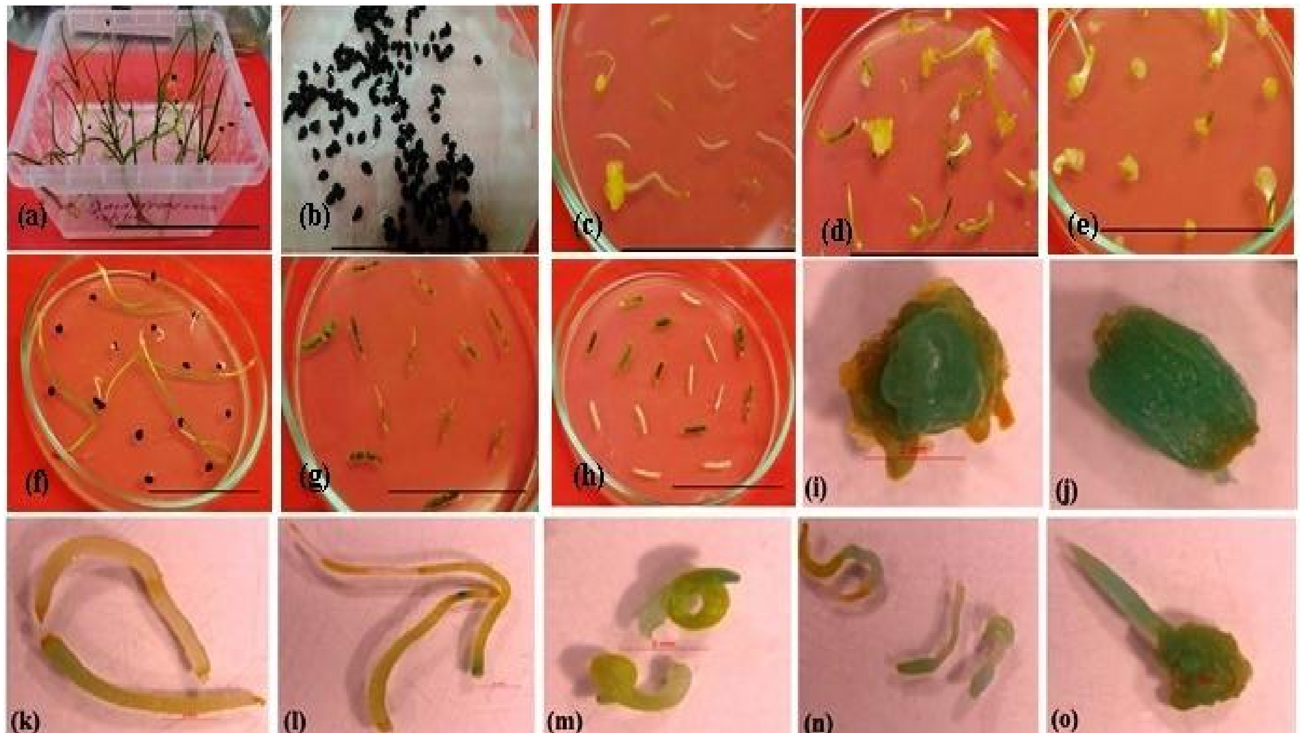


Fig.1: Schematic representation of pBIN19 containing βeta-glucuronidase (*uidA*) and neomycin phosphotransferase (*nptII*) driven by cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (NOS) terminator in between right and left border. The construct has *nptII* gene that encodes resistance to kanamycin, used as a plant selectable marker at 100 mg/l concentration.



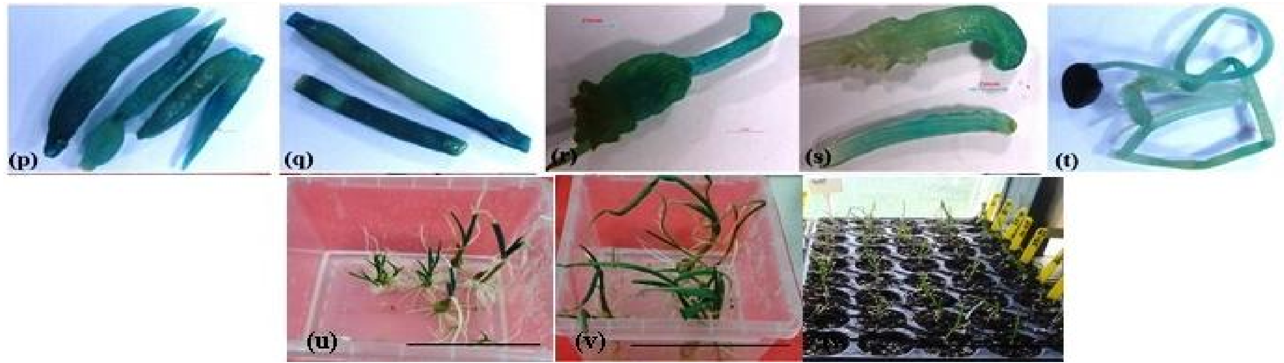


Fig.2: (a). *In vitro* onion plants obtained after one month of growing sterilized seeds on MS basal medium. (b). Pre-soaked onion sterilized seeds on wet sterile filter paper to get mature embryos. (c). Callus formation in root tips. (d). Callus formation in mature embryos. (e). Basal plates showing regeneration. (f). Regeneration in seeds (g). Response of shoot tips on RSM. (h). Response of leaf blades on RSM. (i & j). GUS staining in basal plates of Kral and Sampiyon respectively. (k & l). Root tips of Kral and Sampiyon showing little GUS activity. (m &n). Mature embryos of Kral and Sampiyon showing GUS expression. (o). Root emerging from Kral basal plate showing GUS expression. (p & q). GUS staining in shoot tips and leaf blades respectively. (r & s). Regenerated shoots from Sampiyon and Kral basal plates respectively showing GUS activity. (t). GUS expression in seedling. (u & v). In-vitro putative transgenic plants of Sampiyon and Kral respectively. (w). Transfer of putative transgenic plants in soil.
Bar = 1 cm

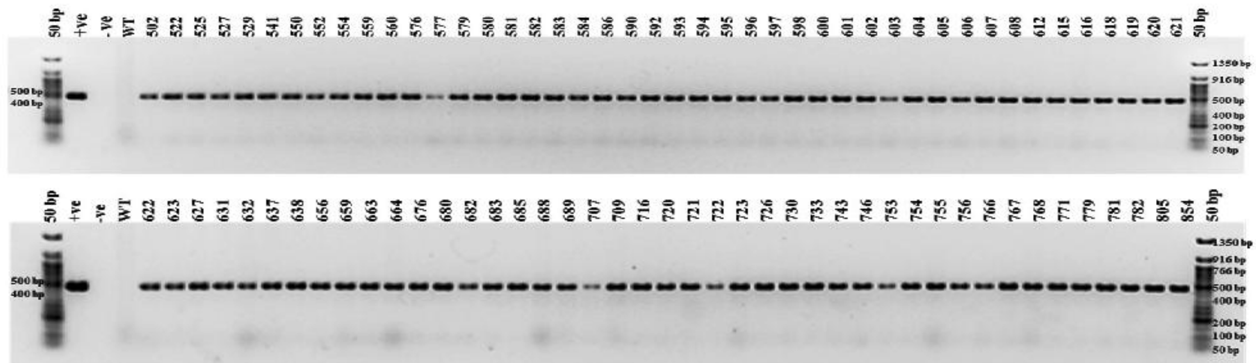


Fig.3: PCR confirmation for the presence of 450 bp fragment of *nptII* gene, 50 bp DNA Ladder; +ve= Positive control (pRD400 Plasmid harboring *nptII* gene); -ve= without DNA control; WT= wild non-transformed DNA, Lane 502-854 putative transgenic plants of Sampiyon and Kral cultivars

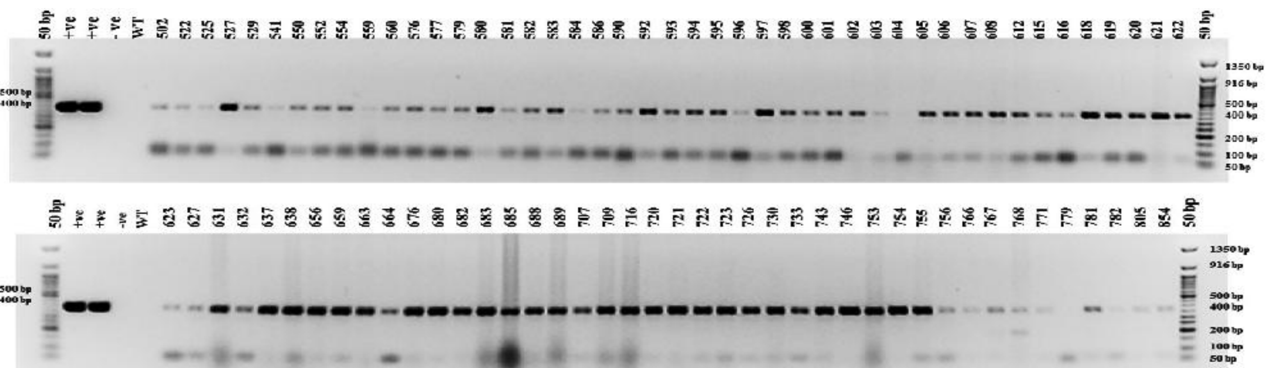


Fig.4: PCR confirmation for the presence of 362 bp fragment of *uidA* gene, 50 bp DNA Ladder; +ve (i) = positive control (pCambia Plasmid); +ve= positive control (pCam-EPSPS Plasmid harboring *uidA* gene); -ve= without DNA control; WT= wild non-transformed DNA; Lane 502-854 putative transgenic plants of Sampiyon and Kral cultivars

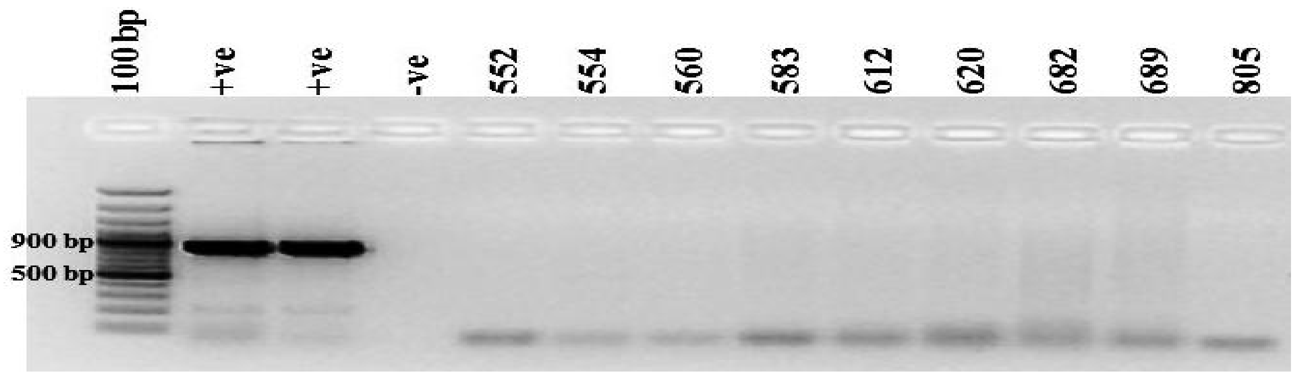


Fig.5: PCR assay for the presence/absence of Chv-A gene (product size: 890 bp) in putative transgenic plants. 100 bp DNA Ladder; +ve = positive control *Agrobacterium* strain LBA4404 harboring PTF-101 plasmid; -ve= non-transformed DNA; Lane 552-805= randomly selected putative transgenic plants of Sampiyon and Kral cultivars showing the absence of 890 bp band

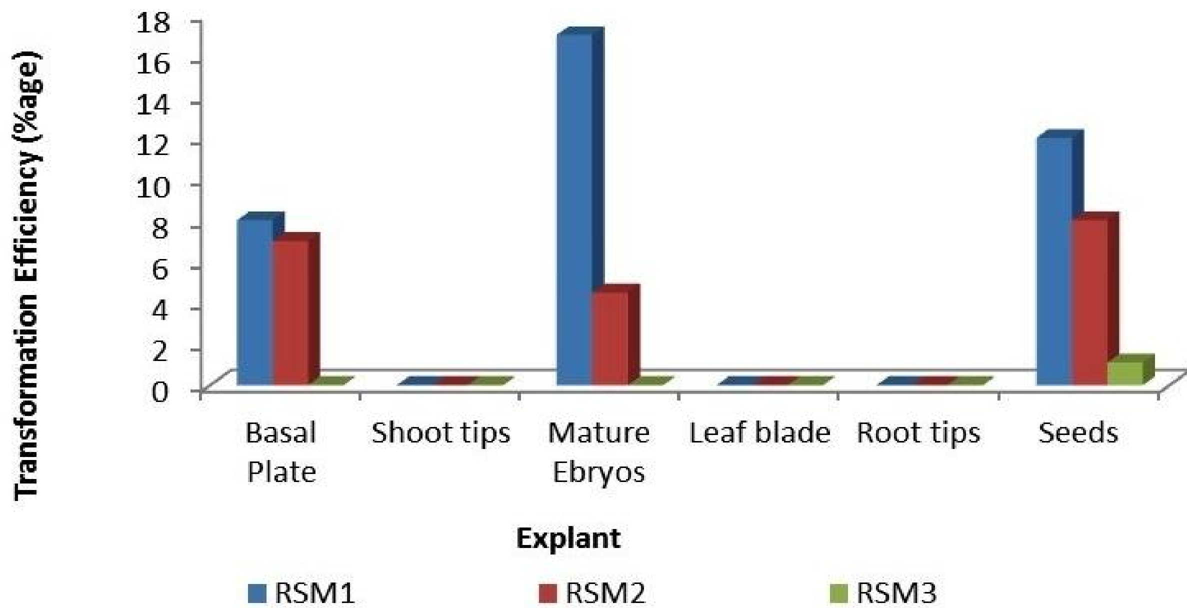


Fig.6: Transformation efficiency of various explants on different media in Kral cultivar

Fig.7: Transformation efficiency of various explants on different media in Sampiyon cultivar

DISCUSSION

The study reports an insight of the factors effecting *Agrobacterium tumefaciens* mediated transformation in onion. Factors affecting callus induction, regeneration ability in onion were optimized, such as response of genotype, type of explants and composition of different plant growth regulators in callus inducing and regeneration media.

Response of Onion Cultivars: Onion being a monocotyledonous crop is perceived to be a difficult crop for genetic transformation (Arumuganathan and Earle, 1991). Onion having large genome size poses less response to transform genetically which may reduce the likelihood of transgene integration at active site of genome and probability to have correct transgene expression may affect (Eady *et al*. 1995a). Due to less response of monocots for *Agrobacterium*-mediated transformation, particle bombardment method was used to transform monocotyledonous crops genetically (Christou *et al.* 1992).

An optimized and functionally stable protocol having a reasonable number of transformants may solve the problem of less responsiveness of monocots for *Agrobacterium*-mediated transformation (Arumuganathan and Earle, 1991). The suitability of LB4404 strain harboring various plasmids for the transformation of monocots (Eady *et al*. 2000) as well as dicots has been already established (Bakhsh *et al.* 2014). Many reports on successful *Agrobacterium*-mediated transformation in cereals have been published. Wheat, rice, sorghum and barley are among those monocot plants that can be efficiently transformed by *Agrobacterium tumefaciens* (Hiei *et al.* 2014; Hiei *et al.* 1997; Hiei *et al.* 1994; Ozawa, 2012).

Present investigation was planned to optimize the factors influencing *Agrobacterium*-mediated transformation efficiency in onion. The plant transformation and regeneration frequency is influenced by genotype, explants and the composition of regeneration media as well as interaction between genotype and media used (Khanna and Raina, 1998). Out of 9 different verities tested, Sampiyon and Kral gave good *in vitro* germination %age. These two varieties were subjected to transformation experiments.

Different onion genotypes responded differently on regeneration medium. There were large differences in their response to regeneration system (Valk *et al.* 1992). Several other reports favored the similar results about response of different genotypes on regeneration media (Kamstaityte and Stanys, 2004; Khar *et al.* 2005; Hailekidan *et al.* 2013). Phillips and Hubstenberger (1987) reported that the hybrids of *Allium fistulosum* x *Allium cepa* were highly responsive to regeneration system. Furthermore, Eady *et al.* (1995b) documented that “Yellow Express” also known as “Sapporo Yellow” onion cultivars gave relatively best response to *in vitro* experiments.

The current research investigated that onion transformation and *in vitro* regeneration system were dependent on the cultivar and explants used. Results showed that the variety “Kral” performed better considering the response of callus induction and further regeneration response. GUS expression was good in Kral as compared to Sampiyon. Out of 87 total onion transgenic plants obtained, 51 were contributed by Kral. Buitveld and Creemers-Molenaar (1994) documented the similar results about genotype and explants dependency in leek transformation and regeneration cultures.

The primary transformants were subjected to PCR analysis to confirm the presence of introduced gene (s). Results showed the amplification of 450 bp and 362 bp product size for *nptII* gene and *uidA* gene respectively, indicating the proper incorporated transgene in onion genome. PCR was conducted to check the *Agrobacterium* contamination in putative transgenic plants. The results exhibited the absence of 890 bp band size which ensured the absence of any agrobacterium contamination. Similar study was done by Nain *et al.* (2005) to confirm the presence of *Agrobacterium* in transgenic plants by amplification of *Agrobacterium* chromosomal genes. A notable number of transgenic escapes were also found in our study. Zheng *et al.* (1998) reported the similar phenomenon that a number of untransformed plants escaped from selection in onion transformation experiments with three-week old callus, induced from mature embryos, as target tissues using *Agrobacterium* and hygromycin as a selection system.

Response of Different Explants: Several initial reports were given on explants dependency on transformation system, callus induction and regeneration cultures by Eady *et al*. (2000), Eady *et al*. (1998a), Eady *et al*. (1995b), Eady *et al*. (1998b), Hussey and Falavigna (1980), Marinangeli *et al*. (2005), Rabinowitch and Brewster (1990), Zheng *et al*. (2001), Zheng *et al*. (2005) and Zheng *et al*. (1998).

In the present study, mature embryos, basal plates and seeds gave good response on callus inducing and regeneration media. The highest transformation frequency obtained was 17% in mature embryos followed by 12% in seeds and 8% in basal plates. Dark green callus observed in shoot tips and leaf blades but these calli did not show response on shoot induction medium. Root tips also showed tendency to develop callus on callus inducing medium. None of positive transgenic plant was obtained from shoot tips, root tips and leaf blades.

Mature embryos could prove efficient explants in onion transformation and regeneration experiments. Similar findings were documented by Buitveld and

Creemers-Molenaar (1994), Shahin and Kaneko (1986) and Valk *et al*. (1992) who conducted experiments using onion mature embryos or seedlings derived calli. The callus derived from embryo showed high regeneration frequency. Zheng *et al*. (2005) and Zheng *et al*. (2001) documented various successful reports on onion transformation and regeneration using mature embryos as experimental material; however more transformation efficiency in our study has been recorded.

Basal discs or basal plates also showed potential to be an efficient explant for onion transformation and *in vitro* regeneration cultures in this study. Hailekidan *et al*. (2013) and Malla *et al*. (2015) used basal plates as explant subjected to DNA delivery system in onion. Viterbo *et al*. (1992) reported that the most efficient callus systems were derived from basal plates and embryos; establishing that basal plate can be competent explant for genetic modification.

The response of seeds in transformation and regeneration was effectual showing good transformation efficiency. From the limited literature, available on *Agrobacterium*-mediated transformation in onion, there is no work done on direct seed transformation of onion. In the current study, sterilized seeds were used as explants in transformation experiments. Our results show that onion seeds can be potential explants for onion transformation experiments being readily available and easy to work. The mean transformation efficiency observed in seeds was as good as in mature embryos and basal plates in a specified composition of regeneration media and genotype used.

Effect of Different Plant Growth Regulators: Although genotype and type of explant are considered as the major determinants of in-vitro plant regeneration response, however, composition of callus inducing medium and regeneration medium is another important factor to deal with. An overall analysis of in-vitro response variations also revealed that there was a significant interaction between the callus inducing medium used for de-differentiation and regeneration selection medium used for re-differentiation (Khar *et al.* 2005). Our study also found out that composition of callus inducing and regeneration media contributed a vital role in successful in-vitro onion regeneration and there was a significant effect of genotype and explants interaction with media used for plant proliferation.

The outcomes of this study revealed that very high concentrations showed negative effects on callus inducing ability that are in agreement with Kamstaityte and Stanys (2004). A good callus induction proportion was observed in both onion varieties (Kral and Sampiyon) when MS medium supplemented with BAP and 2,4-D. When the concentrations of 2,4-D and BAP in medium were increased from 1:10 ratio respectively, it had negative effect on callus induction. Similar type of results and response of growth regulators has been investigated by Ramakrishnan *et al*. (2013) and Zheng *et al*. (1998) also stated that concentration of 2,4-D was the most important factor to optimize to obtain good callus induction and plant regeneration in onion.

Direct regeneration was observed in mature embryos, seeds and basal plates on regeneration medium-1 (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA). When BAP and NAA were used in equal concentrations, mature embryos, seeds and basal plates showed regeneration ability but mean transformation frequencies % were very low on this medium in both varieties. Regenerated shoots were transferred to shoot/root medium (MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 1 mg/l GA3, 500 mg/l). GUS reporter system gave visual identification of transformed callus due to GUS activity (Zheng *et al.* 2005).

The research also revealed that most onion calli usually showed less further proliferation. A good proportion of callus induction was observed in mature embryos followed by basal plates. Root tips also showed callus induction ability. But mostly calli did not respond further on regeneration media. Similar results were documented by Rabinowitch and Brewster (1990).

Conclusions: The results of the study supported the genotype and explants dependency in onion towards genetic manipulation. The variety "Kral" performed better considering the response on callus inducing and regeneration cultures and overall transformation efficiency. Out of 87 total onion transgenic plants obtained, 51 were contributed by Kral. The highest transformation frequency was observed in mature embryos followed by seeds and basal plates. Results of the present study establish that explant selection is important step in onion transformation. Furthermore, seed can also be used as explant for transformation studies. The overall results documented herein may be helpful for the future research regarding introduction of any desired gene in onion.

Authors Contribution: K Amin conducted the experiments, recorded data, wrote her MSc Thesis and initial draft of manuscript that was further read and corrected by A Bakhsh. AF Gökçe secured funding for the research work. A Bakhsh and AF Gökçe supervised the overall research activities together.

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