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EFFECT OF VARIETY AND PLANT GROWTH REGULATORS ON CALLUS PROLIFERATION AND REGENERATION RESPONSE OF THREE TOMATO CULTIVARS (*LYCOPERSICON ESCULENTUM*)

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Abstract

The experiment was conducted to optimize a reproducible protocol for callus induction and regeneration of three tomato cultivars and also to select the cultivar which better perform under *In vitro* conditions for further experimentation. For callus induction hypocotyls and leaf discs were used as explant source. Explants of the tomato seedlings were cultured on MS medium supplemented with different concentrations and combinations of different plant growth regulators (PGRS) for callus proliferation. Callus induction values were significantly influenced by the variety and explant source. In all three tested cultivars maximum callus induction frequency was observed on CIM6 (MS + 0.5 mg/l IAA+2mg/l 2ip). There was also a positive correlation between the treatment and explant source. Here at T6 (2ip 2mg/l, IAA0.5 mg/l) hypocotyls gave its maximum value of (81.8%), followed by theT4 (IAA 1mg/l+kinetin 0.5 mg/l) for hypocotyls (79.48%). To observe the regeneration capacity of three tomato cultivars, 10 Regeneration Media (RM) combinations were tested. The regeneration capacity is significantly influenced by cultivar and explant type. The best regeneration media was found to be RM3 (MS +1.5 mg/l 2ip and 0.5 mg/L IAA) with (72.5-79.22%) regeneration. Among varieties Riogarande showed maximum regeneration capacity (79.22%).

Introduction

Tomato is planted in almost 4 million ha worldwide (James *et al*., 2004). With wide range of adaptability to soil and climate, it is cultivated almost all over the world, It is popular because of its high nutritive value and diversified uses Hundred gram of edible parts of tomato contains 0.9 grams protein, 0.1 gram fat, 3.5 gram carbohydrates, 15-20 calorie energy, 500-1500 IU vitamin "A", 0.1mg thiamine, 0.02 mg riboflavin, 0.6 mg niacin, 20-25 mg vitamin's", 6-9mg calcium and 0.1-0.3 mg iron (Farid Uddin *et al*., 2004).

In tomato genetic transformation with regeneration *In vitro* has been successfully used for genetic improvement (Lindsey 1992). Tolerance to herbicide resistance to pests and diseases, production of edible vaccines or other novel byproducts and quality improvement are the most important goals of genetic plant modification. One of the most important techniques being used now a days is tissue culture for rapid and pathogen free plants. (Averre & Gooding, 2004). Regeneration of whole fertile plants from appropriate tissues *In vitro* is important in plant transformation.

The most important procedure up to date is regeneration through adventitious organogenesis (Van Roekel *et al*., 1993; Frary & Eaarle 1996; Peres *et al*., 2001). *In vitro* plant regeneration has been found to depend on many factors, of which most important are: composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers (Reed 1999). The choice of

an explant with regard to the tissue of origin, developmental stage and physiological state must be determined by goals of the project. Selection of an appropriate culture medium is based upon the nutritional and hormonal requirements of tissues literally cut off from the metabolism of complete plant and varies from species to species.

In vitro culture is used in tomato in different biotechnological applications, production of virus free plants (Moghaieb *et al*., 2004), genetic transformation (Ling *et al*., 1998) and in many fundamental research programme (M Arriliaga *et al*., 2001). Most of the reports about adventitious regeneration in tomato deals with induction of regeneration in hypocotyls or cotyledon explants (Moghaieb *et al*., 2004, Brichkova *et al*., 2002, Raziuddin *et al*., 2004). These *In vitro* seedlings derived plants under aseptic conditions were cut in to small pieces. Many workers reported the hypocotyls (Paranhos *et al.* 1996, Davis *et al*., 1994, Rashid *et al*., 1996, Jatoi *et al*., 1995, Park *et al*., 2003) and leaf disc (Soniya *et al*., 2001, Raj *et al*., 2005, Park *et al*., 2003, Roy *et al*., 2006, Chaudhary *et al*., 2004, Jabeen *et al*., 2005, Raj *et al*., 2005) as explants source. Shoot formation from explants of apical meristem, cotyledons, stems petioles, leaves, anthers and inflorescences has been reported in tomato (Young *et al*, 1987; Branca *et al*., 1990; Compton & Veilleux 1991). Gubis *et al*., 2003 used 6 different types of explants (hypocotyls, cotyledon, epicotyls, leaf petiole and internodes of 13 cultivars of tomato. Takashina *et al*., 1998 reported that the explants nature affected callus induction and regeneration.

De-differentiation of tomato (*Lycopersicum esculentum* Mill) leaf explants into callus followed or not followed by shoot formation is dependent on genotype, culture medium and physiological stage of the donor plant (Guillermo *et al*., 2003).

In the view of the above facts, the present research was designed to evaluate the effect of variety and plant growth regulators in MS medium on Callus proliferation and regeneration from tomato explants.

Materials and Methods

Three varieties of tomato (*Lycopersicon esculentum cv*.) were used. The seeds of Roma Riogarande and money maker were kindly provided by Awan seed center and from National Agriculture Research Centre, Islamabad.

Seeds were surface-sterilized in 0.8 %(v/v) "Clorox" bleach (Sodium hypochlorite) for 15 minutes and rinsed three times (5 min each) with autoclaved distilled water (Franklin & Dixon, 1993). Afterwards the seeds were placed separately in four sterilized Petri dishes containing filter paper (Whatman No.1). Seeds were inoculated in test tubes containing full strength MS medium. Cultures were initially kept in dark for two days at 25 ± 1°C and then maintained under 16h photoperiod at 50 u mol/ m^2/s, with day night temperature at 20°C-25°C.Germinated seedlings served as explant source for tissue culture experiments.

Callus induction and growth: From the 2-3 weeks old *In vitro*, seedlings, hypocotyls (Paranhos *et al.,* 1996) and leaf discs were cut. The hypocotyls and leaf discs were excised of uniform sizes approximately, 1 cm in length. Callus induction responses were assessed for three varieties on 9 media formulations. The hypocotyls were cut into a lower, middle and upper segment. The explants were placed horizontally on the medium surface, leaf discs explants with the adaxial surface in contact with medium. Results were secured according to the presence of callus after 28 days. Basic MS medium with various

concentrations of auxins and cytokinins were used for callus induction. Different concentrations of NAA, BAP, IAA, Kinetin and 2ip were used. As supplement GA3 was also used. GA3 concentration ranged from 1-2mg/l.

Healthy calli were cut into pieces and sub cultured for maintenance onto the same medium, under the same culture conditions to assess the regeneration response.

Regeneration medium: The maintained calli were sub cultured on regeneration media. Ten plant regeneration media, namely RM1 to RM10 (Regeneration Media), were used for identification of regeneration capacity of different cultivars of *Lycopersicon esculentum*.

Rooting medium: As the tomato shoots began to regenerate from calli, they were transferred to rooting media supplemented either with IBA, NAA, or IAA, 0.1 and 0.2 mg/l and the number of shoots that produced roots was recorded after three weeks of incubation. All media contained 3% sucrose with pH adjusted at 5.76 and were solidified with 4g/l of gelrite.

Results and Discussions

In vitro culture is used in tomato in different biotechnological applications, production of virus free plants (Moghaieb *et al*., 1999), genetic transformation (Frary & Earliee 1996; Ling *et al*., 1998) and in many fundamental research programmes (Hanus Fajerska 2000; mArriliaga *et al*., 2001). Most of the reports about adventitious regeneration in tomato deals with induction of regeneration in hypocotyls or cotyledon explants (Asakura *et al*., 1995; Icjimura & Oda 1995; Moghaieb *et al*., 1999). We are also using hypocotyls and leaf discs for establishment of high frequency regeneration in tomato cultivars Roma, Riogarande and Money maker which can be used in future for *Agrobacterium* mediated transformation in these tomato cultivars.

Sterilization is an important step, which affects the callus induction frequency and regeneration. Clorox (Sodium hypochlorite) used as a surface sterilization agent, played an important role in germination of seeds. Seeds of all three varieties Roma, Riogarande and Money maker were treated with different concentrations of Clorox to optimize the level of clorox suitable for *in- vitro* germination. High concentration had an inhibitory effect and seeds become dead whereas when concentration was diluted from 1:0-1:8 the percentage germination of seeds was enhanced. Our results were in line with Chaudhry *et al*., (2001, 2004) and contrary to Reda *et al*., (2004). Reda *et al, 2004* used 70% ethanol followed by 3% Clorox, while Chaudhry *et al*, (2004) used 8% Clorox for the sterilization of seeds.

Seeds were inoculated on MS medium to observe the behavior of varieties for *in vitro* seed germination. Gubis *et al*., (2003) and Raj *et al*., (2005) used half strength MS medium for *in vitro* germination. Moneymaker seeds showed 86.6%. Riogarande 97.2% and Roma; showed 72.5% germination. The results show that Riogarande is the variety that gave the maximum percentage of seed germination. The reason of difference in germination rate might be linked to the genotypes of the varieties. JaeBok *et al.*, (2001) also confirmed these results that germination rate depends upon the genetic basis of the variety.

These *in vitro* seedlings were further used as explants source in callus induction, which leads to regeneration (Fig. 1a,b,c). De-differentiation of tomato (*Lycopersicum esculentum* Mill) leaf explants into callus followed or not followed by shoot formation is dependent on genotype, culture medium and physiological stage of the donor plant(Guillerno *et al*., 2003).

During present studies the callus formation was achieved on MS medium supplemented with IAA, BAP, NAA, GA3, 2ip and Kinetin. Nine Callus Induction Media, viz., T1 to T9 were checked for callus induction, using explant e.g., hypocotyls, leaf discs of three tomato cultivars. Observations in this regard are given in Tables 1 and 2.

In vitro seedlings of 2-3 weeks were used for callus induction (Fig. 1a,b,c). Hu & Philips (2001) also reported to use 18 days old plant for callogenesis and regeneration. Unlike Reda *et al*., (2004) who used 6 days old seedling for callus induction and regeneration. Soniya *et al*., 2001 used leaf explants of *Lycopersicum esculentum* Mill cv.Sakthi from a field grown plant (mother plant).For sterilization she used 70 % ethanol followed by 0.1% Mercuric chlorite.

These *in vitro* seedlings derived plants under aseptic conditions were cut into small pieces. The explants sources were leaf discs and hypocotyls. Many workers reported the hypocotyls (Davis *et al*., 1994, Rashid *et al*., 1995, Jatoi *et al*., 1995, Park *et al*., 2003) and leaf disc (Soniya *et al*., 2001, Raj *et a*l., 2005) as explants source. Gubis *et al*., (2003) used 6 different types of explants (hypocotyls, cotyledon, epicotyls, leaf, petiole and internodes of 13 cultivars of tomato. Takashina *et al.*, 1998 reported that the explants nature effect callus induction and regeneration.

Callus induction and regeneration percentage was a special matter of interest in the studies. Our results were varying on the basis of treatments and genotype of the cultivars. Among the nine combinations of callus induction media T1-T9. T6 (MS plus 0.5 mg/l IAA, and 2mg/ l 2ip) was the most responsive medium. Varieties and explants source varied significantly with different combinations used.

Callus induction values varied significantly among the treatments (Table 1). T6 gave maximum value for the variety Riogarande (80.72%), followed by T5 (GA3 1.5 mg/l and BAP0.5 mg/l) for Riogarande that is (80.67%). Roma gave its maximum value in T6 that is 78.5, followed by T5 that is 77.50. Money maker had its highest value at T6 that is (77.5%) followed by T4 (IAA 0.5 mg/l and Kinetin 0.5 mg/l), that is 72.50%. At T1 (MS salts +vitamins without hormones) all varieties gave there minimum value (.00-.05), followed by T8 that is (NAA 0.5 mg/l, BAP 4mg/l, IAA 0.5 mg/l and 2ip 0.5 mg/l). .Hille *et al*., (1989), Gubis *et al*., (2003), Raj *et al*., (2005) and Park *et al*., (2003) observed that callus is generally induced on medium with high cytokinin to moderate levels of auxin. In our study, also as there was a decrease in the callus induction percentage as BAP concentration was enhanced.

In this study, callus induction value for the two explants used varied significantly with different treatments (Table 2). At T6 the hypocotyls gave the maximum Value that is 81.8, followed by T4 that is 79.48% for the hypocotyls followed by T5 that is 79.44% for hypocotyls. Leaf discs gave there maximum value at T6 (76%), followed by T4 that is 72.33%. Minimum values for callus induction in explants were at T1 followed by T8.

The probable reason for more callus induction percentage might be the requirement of moderate concentrations of auxins combined with relatively higher levels of cytokinins (Hille *et al.*, 1989). Capote *et al*., (2000) also obtained more callus induction by combination of Auxin and relatively high level of Cytokinin.Therefore, higher levels of auxins as well as higher levels of cytokinins gave poor callus response and if the callus is formed is completely brown or black. On the other hand CIM1 with lower level of auxins also could not give good results for callus induction. These results are also in line with Jatoi *et al*., (2001) who obtained 100% callus induction in medium with 10 µM BAP and 0.1 µM/l IAA. Park *et al*., (2003) used 2mg/l of zeatin alongwith 0.1mg/l of IAA in the transformation protocol, in this experiment zeatin was replaced by 2ip. Reda *et al*., (2004) used higher auxin and low level of cytokinin for callus induction. She used 2mg/l of 2,4-D and 0.25 mg/l of kinetin. Soniya *et al*, (2001) used 8.88 uM of BA and 4.13 uM piclogram for callus induction.

The results indicated that the Riogarande produced the maximum average calli (85-95%), which was significantly higher than all the other varieties used during the present experiment (Fig. 1d). The callus induction in Roma was 75-80% on the average, which is higher than that in Money Maker. These differences in callus percentage may be linked to their genotypes. Genotypic constitution has an effect on callus induction and regeneration (El-Farah *et al*., 1993). These results are also confirmed by Lu *et al*., (1997) who observed that different genotypes differ significantly in callus induction and regeneration.

Within same variety, on all media combinations the results were different in all three varieties while on same media combination among three cultivars there are some non-significant results. This shows that media combinations or hormonal balance has strong effect on callus induction as compared to genetic variation.

The maintained calli were subcultured on regeneration media. Different hormonal combinations were used in Regeneration Media (RM), namely RM1 to RM10. The results are shown in Table 3, 4, 5 and 6. According to the results, all 10 treatments of media combinations showed different behavior in regeneration response except RM1 (without growth hormone) and RM10 (2mg/l IAA only without Cytokinin), therefore these two media combinations showed negligible regeneration.

Varieties and explant source showed significantly different results for different combination of media used (Table 5). At T3 (2ip 1.5 mg/l and IAA 0.5 mg/l) Riogarande gave its maximum value that is 79.22, followed by Roma at T3 that is 76. Money maker gave its maximum value at T3 that is 72.5.After T3 Riogarande gave its maximum value at T2 (2mg/l BAP and 0.5 mg/l of IAA) that is 75%. At T9 (0.5 mg/l of IAA and1mg/l of 2ip) Roma gave its second highest value that is 67, followed by T7 (1mg/l of IAA, 1mg/l of 2ip and 0.5 mg/l of Kinetin) that is 60%.

Regeneration values also varied significantly with different explants used for various treatments (Table 5). At T3 both hypocotyls and leaf disc showed maximum regeneration value that is 79.45 for leaf disc and 72.33 for hypocotyls followed by T9 that is 53.33% for leaf discs and 60 for hypocotyls.

There was also significant difference for different varieties used and different explant used for different regeneration medias used. There was significant difference in the interaction of the treatment explant source and varieties used (Table 3). Leaf discs and the Riogarande gave their maximum value at T3 that is 83% and 75% for hypocotyls, followed by 80% for leaf discs and 72% for hypocotyls for Roma. For money maker 75% in case of leaf discs and 70% for hypocotyls.

The presence of high cytokinins with low or equal amount of auxins was also confirmed by Gubis *et al*., (2003), who observed the regeneration capacity of six types of explants in 13 tomato (*L. esculentum*). Soniya *et al*., (2001) used only 17.7uM of BA for regeneration. Raj *et al*., (2005) used low levels of auxin and cytokinin for regeneration of leaf explants of the Pusa Ruby tomato e.g., 0.1 mg/l of IAA and 0.1mg/l of zeatin. Chaudhary *et al*., (2004) obtained the regeneration percentage of 45.8% and 30.8% for the hypocotyls and leaf discs respectively by using relatively higher level of auxin and higher amount of cytokinin e.g. IAA 2mg/l, BAP 5mg/l, NAA 2mg/l and kinetin 4mg/l. The best results were obtained when explants were cultured on a regeneration medium containing 2 mg 2ip/litre and 0.1 mg IAA/litre. The presence of equal amounts of auxin and cytokinin in the regeneration media, were confirmed by Botau *et al.,* (2002). They explored that cotyledons and hypocotyls of tomato cultivars, cultured on MS media supplemented with different hormonal combinations and the best results were obtained in the medium supplemented with 1 mg NAA/litre+1 mg BAP/litre. Chandel & Katiyar (2000) tested different combinations of phytohormones , among them the MS medium

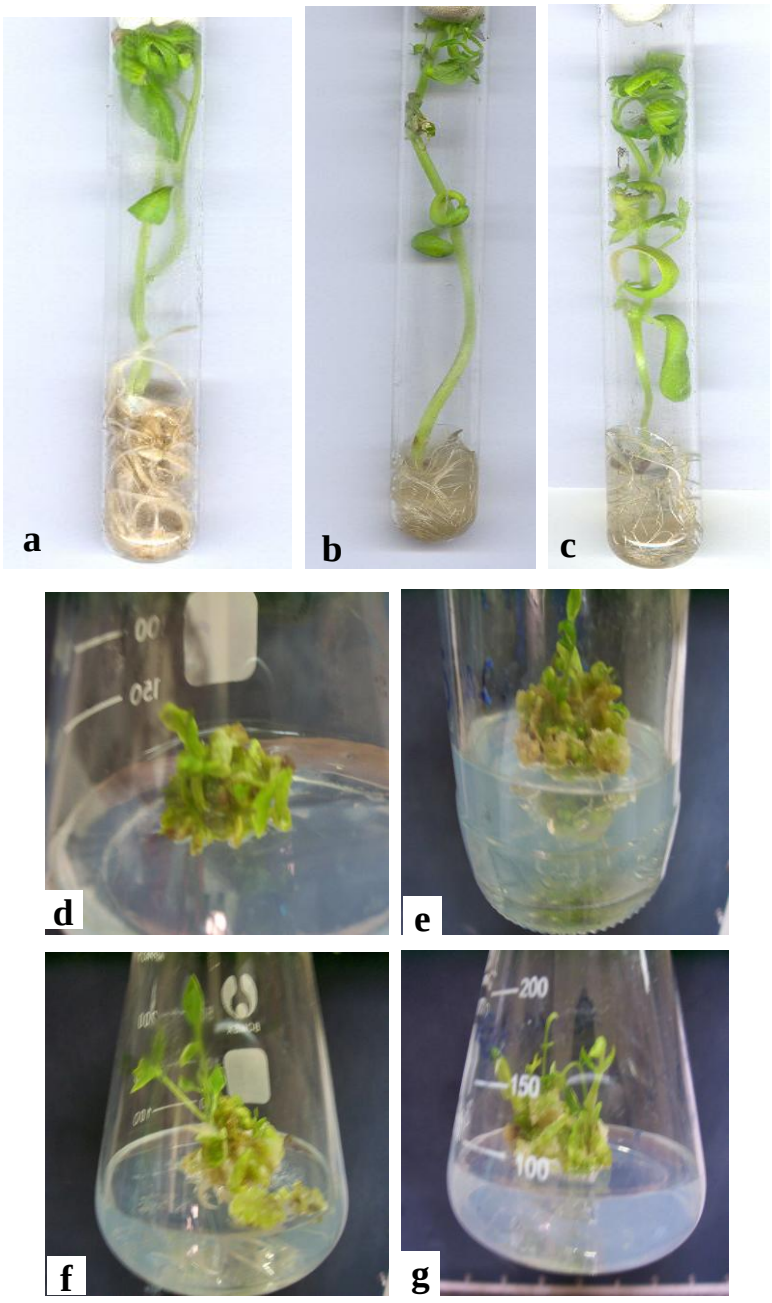

Fig. 1. *In vitro* seedlings of *Lycopersicon esculentum*. (a) Roma, (b) Riogarande, (c) Money maker, (d) Hypocotyls derived calli of Riogarande and (e) Money maker (f) leaf disc derived calli of Roma and (g) Riogarande showing green spotting and regeneration.

Table 5. Regeneration response of three different varieties by using two different explants. (Variety*Explant).

Variety	Riogarande	Roma	Moneymaker	Explant means
Hypocotyl	35.56 BC	34.47 C	30.23 D	33.42
Leaf disc	41.312 A	37.45 B	31.1 D	36.62
Variety means	38.44	35.96	30.67	

Figure with same letter showed the non significant results and with different letters showed significant results.

Table 6. Regeneration response of two different explants of three tomato cultivars on different media combinations. (Treatment*Variety* Explant.

Treatments	Riogarande		Roma		Moneymaker	
	L.D	Hypo	L.D	Hypo	L.D	Hypo
T1	.01 T	.00 T	.01 T	.01 T	.001 T	.001 T
T2	65 EF	85 A	40 JK	30 LMN	30 LMN	20 OPO
T3	83 AB	75 BCD	80 ABC	72 CDE	75 BCD	70 DE
T4	45 IJ	58 FGH	35 KLM	40 JK	30 LMN	28 MNO
T5	30 LMN	42 IJK	30 LMN	28 MNO	24 NOP	29 LMNO
T6	28 MNO	30 LMN	10 RS	25 NO	15 PQR	12 QRS
T7	35 JLM	38 JKL	50 HI	70 DE	30 LMN	27 MNO
T8	20 OPQ	25 NO	30 LMN	35 KLM	40 JK	60 FG
T9	45 IJ	55 GH	65 EF	70 DE	50 HI	55 GH
T10	4.15 ST	5.12 ST	4.7 ST	4.5 ST	8.2 RST	10 RS

Figure with same letter showed the non-significant results and with different letters showed significant results.
*Hormones used for Regeneration medium
RM_1 MS without hormone
RM_2 0.5 mg/l BAP and 2 mg/l GA3
RM_3 0.5 mg/l 2ip and 2 mg/l GA3
RM_4 2 mg/l BAP
RM_5 NAA0.5 mg/l and BAP 5mg/l
RM_6 1 mg/l BAP and 1 mg/l Kinetin
RM_7 0.5 mg/l NAA, 0.5 mg/l and 1.5 mg/l Kinetin
RM_8 1 mg/l IAA and 1 mg/l2ip and 3mg/lKinetin
RM_9 1.5 mg/l IAA and 0.5 mg/l 2ip
RM_{10} 2 mg/l IAA and 0.5 mg/l 2ip

with 1.5 mg BAP and 1.5 mg IAA/litre was the most responsive for regeneration. The presence of two cytokinins in RM3 (MS with BAP 2 mg/l and Kinetin 1 mg/l), showing results was also indicated by Brichkova *et al*., (2002) who observed that presence of two cytokinins (BAP at 5.0 mg and Zeatin at 1.0 mg/litre) in medium contributed to plant regeneration in 74% of Vezha explants and in 89% of L-1932 explants. RM10 showed the poorest results because of the absence of Cytokinin. Among varieties Riogarande showed the maximum regeneration capacity (40 %) (Fig. 2C). All the three varieties showed difference in their regeneration capacity. Moneymaker showed minimum average regeneration capability (75%). These differences might be due different genotypes. These results are close to that of Lu *et al*., (1997), who studied the effects of tomato genotype; explant source and culture medium growth regulator composition on callus formation and plantlet regeneration rates using 2 cultivars. They found that genotype had significant effect on callus induction and regeneration. Mirghis *et al.,* (1995) observed that the *in vitro* reactions of the genotypes differed significantly and were dependent on the culture media. Nandakumar *et al*., (1991) noted that callus formation and regeneration varied with the genotype.

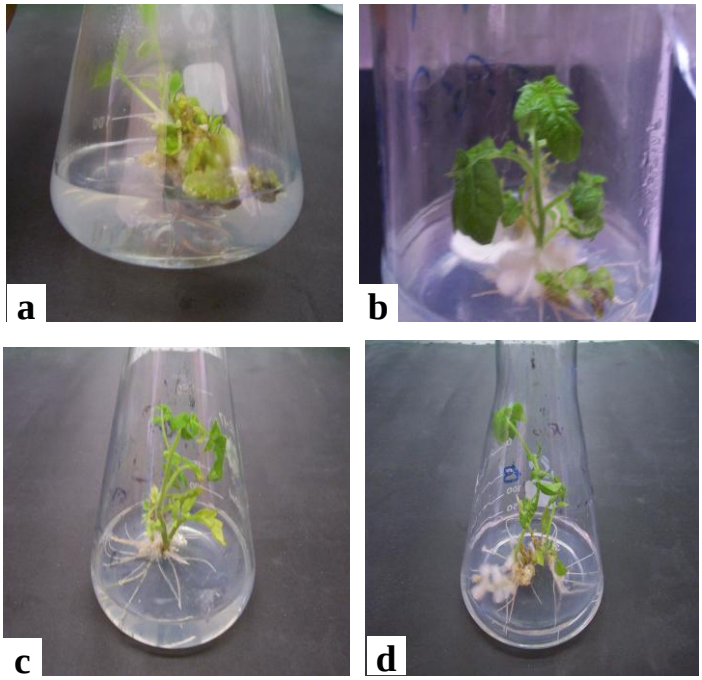

Fig. 2. (a) Leaf disc derived callus induction leading to regeneration in Money maker. (b) Regenerated shoot of money maker placed in rooting medium, (c) Regenerated plant of Riogarande in rooting medium, (d) Regenerated plant of Roma present in rooting medium.

Auxins and cytokinins are involved in cell division and elongation, while; cytokinins help in the process of differentiation. So appropriate concentration of these growth hormones is necessary for cell division and differentiation. During the present study, it was revealed that the varieties varied significantly for their regeneration capacity on the similar medium. Roma and moneymaker showed minimum regeneration in almost all media combinations (Fig. 2a,b,c). This might be due to their genetic differences.

Plant establishment in the soil: Thirty rooted plants derived from each of the hypocotyls and the leaf discs after one week of root formation were shifted into small pots of compost in the glass house. They were covered with the polythene bag for 10-12 days to control the temperature and humidity, and were watered at 4-5 days intervals. About 80-90% of the plants survived in the soil and all survivors flowered normally. About 85% of the seeds collected from these plants were viable

Conclusion

For callus induction study, different Callus Induction Media (CIM) combinations tested on tomato cultivars, all three varieties showed maximum callus induction percentages on T4 (MS + 0.5 mg/lBAP, 2mg/l GA3, 0.5 mg/l NAA). Riogarande had maximum callus induction (80.72%).

From ten Regeneration combinations tested, the best regeneration media was found to be RM3 (MS +1.5 mg/l 2ipand 0.5 mg/l IAA). Among varieties Riogarande showed maximum regeneration capacity 79.2%. All the media combinations showed significantly different results for regeneration. Also the varieties showed significantly different results within the same treatment. All the treatments showed significantly different response to two different explant, used.

Establishment of the high frequency regeneration system is a prerequisite for the genetic transformation. The result of the present study will serve as a basis for efficient transference of desired characters through genetic transformation procedures. The result of the present finding Riogarande cultivar among three varieties used is recommended for Genetic Transformation as being more responsive to the standardized conditions.

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