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FACILE PLANT REGENERATION FROM TOMATO LEAVES INDUCED WITH SPECTINOMYCIN

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Abstract

Several studies have been conducted to find a combination of hormones conducive to improve regeneration from different explant tissues, particularly from leaves of tomato, and various responses were recorded to use in experiments. Major focus was to regenerate maximum number of shoots from tomato leaves C*v.* Moneymaker, Packet, Nagina and Aroma. Depending on the cultivar used, shoot regeneration varied (Moneymaker ≥ Packet ≥ Nagina ≥ Aroma) on RMOT medium (MS salts supplemented with 1mg/L Zeatin and 1mg/L IAA). In addition, several other media were used to compare regeneration efficiencies including RMOP medium containing MS salts supplemented with 0.1mg/L NAA, 1mg/L BAP, 1mg/L thiamine and 100mg/L Myo-inositol. In cultures where negative factors were eliminated, shoot regeneration reached to an average of 9 plants on RMOT medium from each leaf section of 3x3 mm size of cultivar Moneymaker.

Introduction

The explant tissues of tomato from different organs are totipotent to regenerate into plants either through callogenesis or organogenesis however their morphogenic responses are affected by different components of the culture media. Hence, it is important to evaluate their effects on plant regeneration. Tomato is an important crop species and is being used as a model plant in molecular studies. In addition to a kitchen plant, it is considered as a source of economic traits for example, resistance to insect-pests and various diseases (Rick & Yoder, 1988). Particularly after the successful chloroplast transformation, it is being considered as a model plant to express pharmaceuticals, develop edible vaccines and produce cost-effective therapeutics (Ruf *et al*., 2001; Daniell *et al*., 2002; Daniell & Khan, 2003). Different explant tissues like cotyledons, hypocotyl, embryos, ovules and protoplasts of tomato have been used to regenerate plants (Zapata & Sink, 1981; Koblitz & Koblitz, 1982; Uddin & Berry, 1988; Chen & Adachi, 1994; 1996; 1998; Gill *et al.,* 1995; Newman *et al*., 1996; Ling *et al*., 1998; Costa *et al*., 2000). As for as the development of transgenic plants is concerned only cotyledons are used successfully (Ling *et al*., 1998). Recently, fully expanded leaves are used to transform chloroplasts (Ruf *et al*., 2001). Nevertheless, the regeneration response was very poor. In this study antibiotic-resistant calli were subcultured for a long period for proliferation and then shoots were regenerated (Ruf *et al*., 2001). Keeping in view these factors, aim of our study was to develop efficient and reducible regeneration system for leaves, improving transformation efficiencies in tomato. We have developed efficient regeneration system from tomato leaves comparable to tobacco.

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Materials and Methods

Plant material and *In vitro* propagation: Sterile tomato plants (*Lycopersicon esculentum Cv*. Moneymaker, Packet, Nagina and Roma) were raised in Jars from surface-sterilized seeds germinated on MSO medium. This medium contains MS salts (4.3 g/l), Glucose (6-7 g/l) and Phytagel (2.6 g/l). Seeds of four tomato varieties (Moneymaker, Packet, Nagina and Roma) were surface sterilized using 70% (v/v) ethanol for 2 minutes and 3.5% (v/v) Sodium hypochlorite for 3 hours, followed by rinsing four times in sterilized distilled water leaving the final change of water shaking at 25°C overnight. Prior to placing seeds on MSO medium, seeds were rinsed for additional 6-8 times with autoclaved distilled water and grown aseptically under controlled low light (30µmole $m^{-2}s^{-1}$) conditions at 25˚C. For about 400 explants, about 100 seeds were germinated.

Explant preparation and plant regeneration: Leaf sections of 3x3mm size prepared from leaves of four to six weeks old sterile plants grown in culture jars under controlled conditions were placed on to the surface of medium containing MS salts (0.44% w/v), phytagel (2.6 g/L) and sucrose (20 g/L) with different combinations of hormones and vitamins for all four varieties of tomato. Media used for efficient regeneration were a) 1mg/L zeatin, 1mg/L IAA and Nitsch vitamins b) 1mg/L zeatin, 0.1mg/L IAA and B5 vitamins c) 1mg/L zeatin, 1mg/L IAA and B5 vitamins. d) 1mg/L zeatin, 1.5mg/L IAA and B5 vitamins. e) 1mg/L zeatin, 2mg/L IAA and B5 vitamins. f) 0.1mg/L NAA, 1mg/L BAP, 1mg/L thiamine and 100mg/L Myo-inositol. g) zeatin 1mg/L, 0.1mg/L NAA and 1mg/L BAP. h) zeatin 1mg/L and 0.1mg/L NAA.

Rooting and plant hardening: Shoots regenerated on different regeneration media were excised with sharp blades and placed on medium containing MS salts 0.44% (w/v), sucrose 2% (w/v), phytagel 2.6 g/L (Difco) of pH 6.0 before autoclaving for rooting. Once roots were established plants were transferred to pots containing peat moss and vermiculite and after sprinkling water were covered with polythene bags to avoid evaporation as well as transpiration that were removed after making holes each day till 7 to 8 days.

Results and Discussion

***In vitro* propagation and plant development:** Sterile plants were developed from sterilized seeds on MSO medium under controlled growth conditions. Seedlings raised were subcultured on same medium by making small cuttings carrying single bud on each cutting. Two different carbon sources were used to get proliferated leaves namely, glucose and sucrose. Plant growth was delayed on MSO medium containing sucrose but the leaves were well proliferated and succulent whereas fast growth was observed on same medium containing glucose with B5 vitamins.

***In vitro* regeneration and shoot development:** Regeneration from leaf sections on medium where MS salts was supplemented with 1mg/L zeatin, 1mg/L IAA and B5 vitamins was started within 3 weeks and shoots remained proliferating on the same medium. These shoots were lush green in color as compared to those obtained on medium where MS salts were supplemented with 1mg/L zeatin, 1mg/L IAA and Nitsch vitamins.

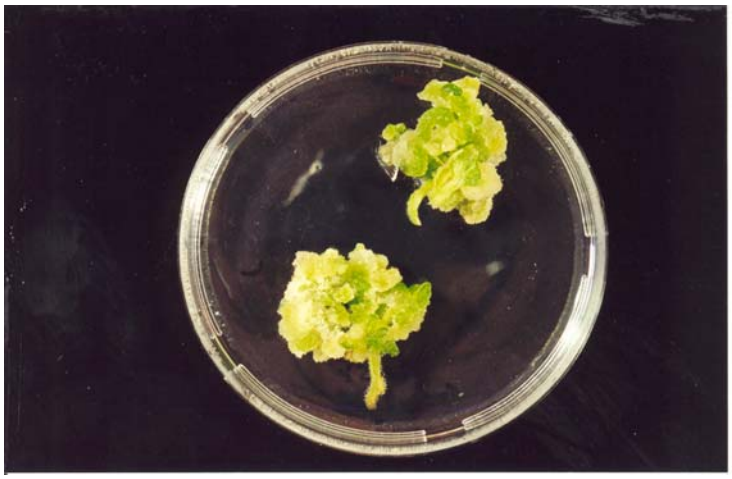

Fig. 1. Callus induction, proliferation and regeneration from cotyledons of tomato.

Several studies have been carried out in various laboratories to regenerate plants from cotyledons to transform cells using *Agrobacterium*-mediated transformation protocols however, for chloroplast transformation the tobacco regeneration medium RMOP (Svab & Maliga, 1993; Khan & Maliga, 1999; Bock & Khan, 2004) was used to regenerate plants from leaves (Ruf *et al*., 2001) following biolistic transformation method (Khan, 2001; Khan *et al*., 2005). Using tobacco regeneration medium RMOP instead of getting shoots directly on leaf sections calli were produced from cut margins of leaves and were kept proliferating on same medium for next couple of months. On transfer to fresh medium (MS salts 0.2mg/L IAA and 3mg/L BAP) proliferation of calli and regeneration into shoots was obtained (Ruf *et al*., 2001). This procedure is known for direct regeneration from leaves in tobacco as shoots directly appear on cultured leaf sections (Svab & Maliga, 1993; Khan & Maliga, 1999), however it appeared to be a two steps regeneration method, first callus induction and second callus proliferation and regeneration in tomato (Fig. 1; Ruf *et al*., 2001). In our studies, we have used different cultivars for direct regeneration of shoots from cultured cotyledons and leaves on media with different hormone combinations and direct regeneration of shoots of *Cv*. Moneymaker on a medium containing 1mg/L zeatin and 1mg/L IAA with B5 vitamins obtained from leaves. It has been observed that low light conditions are conducive for efficient regeneration of plants from leaves irrespective to the medium and cultivar used.

Based on media composition, more callus formation and less regeneration was observed on medium where MS salts were supplemented with 1mg/L Zeatin and 0.1mg/L IAA from leaves similar to cotyledons however, very little callus formation with several shoots was observed on medium containing 1mg/L IAA instead of 0.1mg/L. In addition, it was observed that callus formation starts on cut margins of leaves on all media except where NAA as well as NAA and BAP supplements were used. On these combinations embryos appeared all over the leaf surface (Fig. 2) however regeneration was delayed that was obtained on transfer to regeneration medium as reported in results section. Similar response as regard of embryogenesis was obtained by Zapata & Sink (1981) from micro-callus masses on the medium supplemented with 1mg/L NAA and 1mg/L KIN for

the induction of somatic embryos. When 2, 4-D was replaced with NAA and KIN in the culture medium, no somatic embryogenesis occurred. In contrast, Chen & Adachi (1994, 1998) used zeatin of concentrations 1 mg/L combined with 0.2 mg/L gibberellic acid (GA_3) or 0.5 mg/L Indole acetic acid (IAA) for callus proliferation and subsequently 1, 2 and 3 mg/L zeatin for induction of somatic embryos. Direct somatic embryogenesis has been induced in immature zygotic embryos (Chen & Adachi, 1996, 1998; Uddin & Berry, 1988). Somatic embryos were derived from the hypocotyl and from cotyledons (Uddin & Berry, 1988) in these cases however, we obtained embryos not only on medium where 1mg/L IAA was replaced with 0.1mg/L NAA and 1mg/L BAP also where NAA alone was used to replace IAA in RMOT medium. Direct shoots developed on RMOT medium as concentration of IAA was increased from 0.1 to 2mg/L, and optimum combination with more number of shoots and very little embryogenic callus was 1mg/L zeatin and 1mg/L IAA along with B5 vitamins.

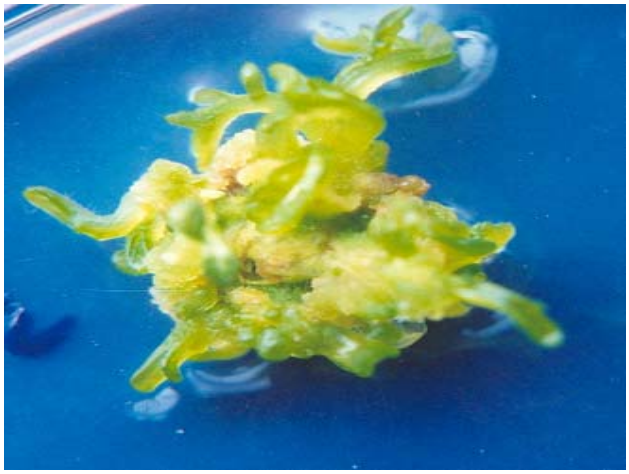

Fig. 2. Regeneration from tomato leaf section.

Regeneration suppression variability using antibiotic spectinomycin: Shoot development was observed on leaf sections cultured onto a MS medium supplemented with 1mg/L zeatin, 1mg/L IAA and B5 vitamins) containing spectinomycin concentrations 10, 20, 25, 30 and 35mg/L (Fig. 3a). Development of shoots was observed on spectinomycin-containing (10 to 35mg/L) medium however, only small calli produced from cut margins of leaf sections that reduced gradually at concentrations of 40, 45 and 50mg/L of spectinomycin and was completely blocked from 75mg/L to onward concentrations (Fig. 3b). Nevertheless, maximum number of shoots was obtained where 15mg/L spectinomycin was added to the medium. In contrast, no regeneration was observed from leaf sections cultured on RMOP medium (MS salts containing 0.1mg/L NAA, 1mg/L BAP, 1mg/L thiamine and 100mg/L Myo-inositol) supplemented with spectinomycin of 10, 20, 25, 30, 35, 40, 45,50, 75, 100, 200, 300, 400 and 500mg/L however, callus formation was observed on concentrations 10 to 200 mg/L spectinomycin which was completely blocked afterward. These calli were proliferated and shoots regenerated on medium where MS salts were supplemented with 0.2mg/L IAA and

2mg/L zeatin but regeneration was poor compared to direct regeneration on medium where MS salts were supplemented with 1mg/L zeatin, 1mg/L IAA and B5 vitamins. The fully regenerated shoots were excised and placed on Murashige & Skoog (1962) medium for roots development. The plants with fully developed roots were shifted into the pot for hardening.

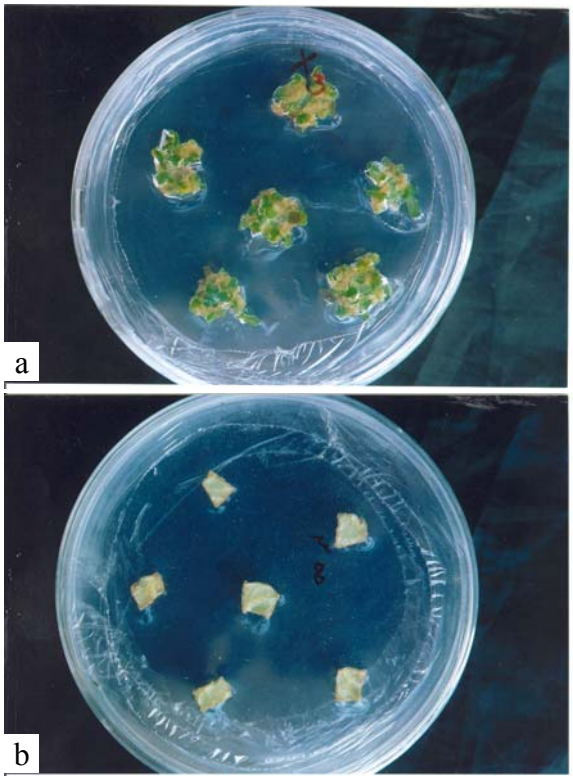

Fig. 3. Spectinomycin effectiveness to block regeneration from tomato leaf sections (a) At a concentration of 15mg/L of spectinomycin maximum number of shoots remained were 9 on average basis from each leaf section. (b) Regeneration was blocked completely on medium containing spectinomycin at concentrations from 75 to 500mg/L

Tomato has been proposed to be a model edible plant to overexpress pharmaceuticals in chromoplasts to provide cost-effective therapeutics in coming few years. Our efficient regeneration system for leaves will facilitate chloroplast transformation in tomato with high efficiencies.

References

Bock, R. and M. S. Khan. 2004. Taming plastids for a green future. *Trends in Biotechnol.,* 22: 311-318.

Chen, L.Z. and T. Adachi. 1994. Plant regeneration via somatic embryogenesis from cotyledon protoplasts of tomato (*Lycopersicon esculentum* Mill.). *Breeding Science,* 44: 257-262.

Chen, L.Z. and T. Adachi. 1996. Efficient hybridisation between *Lycopersicon esculentum* and *L. peruvianum* via 'embryo rescue' and *In vitro* propagation. *Plant Breeding,* 115: 251-256.

Chen, L.Z. and T. Adachi. 1998. Protoplast fusion between *Lycopersicon esculentum* and *L. peruvianum*-complex: somatic embryogenesis, plant regeneration and morphology. *Plant Cell Reports,* 17: 508-514.

Costa, M.G.C., F.T.S.Nogueira, M.L. Figueira, W.C. Otoni, S.H. Brommonschenkel and P.R. Cecon. 2000. Influence of the antibiotic timentin on plant regeneration of tomato (*Lycopersicon esculentum* Mill.) cultivars. *Plant Cell Reports,* 19: 327-332.

Daniell, H. and M.S. Khan. 2003. Engineering the chloroplast genome for biotechnology applications. N. Stewart ed. *Transgenic Plants: Current Innovations and Future Trends,* Chapter 5 of 28 pages, Horizon press UK.

Daniell, H., M.S. Khan and L. Allison. 2002. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends in Plant Science,* 7: 84-91.

Gill, R., K.A. Malik, M.H.M. Sanago and P.K. Saxena. 1995. Somatic embryogenesis and plant regeneration from seedling cultures of tomato (*Lycopersicon esculentum* Mill.). *Journal of Plant Physiology,* 147: 273-276.

Khan, M.S. 2001. Utilizing heterologous promoters to express green fluorescent protein from jellyfish in tobacco chloroplasts. *Pak. J. Bot*., 33: 43-52.

Khan, M.S. and P. Maliga. 1999. Fluorescent antibiotic resistant markers for tracking plastid transformation in higher plants. *Nature Biotech.,* 17: 910-916.

Khan, M.S., A.M. Khalid and K.A. Malik. 2005. Phage phiC31 integrase: a new tool in Plastid genome engineering. *Trends in Plant Science,* 10: 1-3.

Koblitz, H. and D. Koblitz. 1982. Experiments on tissue culture in the genus *Lycopersicon* Miller-mesophyll protoplast regeneration to plants in *Lycopersicon esculentum* cv. "Nadja". *Plant Cell Report,* 1: 143-146.

Ling, H-Q., D. Kriseleitand and M.W. Ganal. 1998. Effect of ticarillin/potassium elavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell Report,* 17: 843-847.

Newman, P.G., Krishnaraj, S. and P.K. Saxena. 1996. Regeneration of tomato (*Lycopersicon esculentum* Mill): Somatic embryogenesis and shoot organogenesis from hypocotyl explants induced with 6-benzyladenine. *International Journal of Plant Sciences,* 157: 554-560.

Rick, C.M. and J.I. Yoder. 1988. Classical and molecular genetics of tomato: highlights and perspectives. *Annu. Rev. Genet*., 22: 281-300.

Ruf, S., M. Hermann, I.J. Berfr, H. Carrer and R. Bock. 2001. Stable genetic transformation of tomato plastids and expression of foreign protein in fruit. *Nature Biotech*., 19: 870-875.

Svab, Z. and P. Maliga. 1993. High frequency of plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl. Acad. Sci., USA* 90: 913-917.

Uddin, M.R. and S.Z. Berry. 1988. Investigations on the somatic embryogenesis in tomato. *HortScience,* 23: 755.

Zapata, F.J. and K.C. Sink. 1981. Somatic embryogenesis from *Lycopersicon peruvianum* leaf mesophyll protoplasts. *Theoretical and Applied Genetics*, 59, 265-268.

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