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EFFECT OF DIFFERENT BACTERIAL DILUTIONS ON TRANSFORMATION EFFICIENCY OF HOT CHILLI (*CAPSICUM FRUTESCENS* L.) VARIETIES

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Abstract

A reproducible and efficient protocol for two varieties of chilli (*Capsicum frutescens* L.) varieties Nepali and NARC-IV using different bacterial dilutions was optimized to assess its effect on transformation efficiency. The hypocotyl segments were inoculated with *Agrobacterium tumefaciens* strain EHA 101 harboring binary vector pTCL5. Plant response in culture was highly dependent on infection of hypocotyls with different bacterial densities. Proliferation and differentiation of calli forming plantlet as well as percentage transformation efficiency was strongly affected by the bacterial dilutions. Drastic increase in plant production from calli along with transformation efficiency was achieved at low bacterial density as compared to high density which in consequences brought about the necrosis and death of calli.

Introduction

Chilli belonging to genus *Capsicum* and family Solanaceae is one of the most important cash crop of the Southern parts of Pakistan. The production of chilli varieties is limited due to prevalence of diseases like Bacterial wilt (*Ralstonia solanacearum*) and Root rot (*Colletotrichum capsici*). In cultivated varieties or sexually compatible species, genetic transfer is considered as an efficient method of introducing resistance. Transformation of dicotyledonous plants *via Agrobacterium tumefaciens* is still far from routine and there is not a universal protocol suitable to transform genetically different cultivars within species (Romero *et al*., 2001; Lee *et al*., 2004). Application of cell and molecular biology techniques for the genetic improvement of this crop has been limited because of difficulties in plant regeneration and ultimately in linking regeneration with transformation (Lim *et al*., 1999). Regeneration in many different varieties of chilli was reported *via* shoot organogenesis in cotyledon and hypocotyl explants. During last few years, transformation using *Agrobacterium tumefaciens* has been reported in sweet pepper and hot chilli (Li *et al*., 2003; Lee *et al*., 2004; Delis *et al*., 2005). However, no such success has been obtained because the protocols failed to reproduce the results. Dilution of *Agrobacterium* culture prior to co-cultivation is the key for achieving increased transformation efficiency and effective control of bacterial overgrowth (Mercuri *et al*., 2000). In order to demonstrate that the recovery of transgenic plants of chilli requires the use of appropriate bacterial dilutions for improved transformation efficiency, different bacterial dilutions were used to achieve stable transformation of *Agrobacterium tumefaciens* strain EHA 101 harboring binary vector pTCL5 having *Xa21* gene for resistance to bacterial wilt, with further confirmation of *gus* gene by GUS assay.

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Materials and Methods

Explant source: The certified seeds of chilli varieties Nepali and NARC-IV were taken from Vegetable Section of Horticulture Sciences Program (HSP), National Agriculture Research Centre (NARC), Islamabad. For transformation, hypocotyls excised from healthy *In vitro* grown seedlings were used.

Bacterial strain and culture: Transformation was carried out through *Agrobacterium tumefaciens* strain EHA101 containing pTCL5 plasmid (Agriculture Biotechnology Program, NARC). It is a binary vector containing *Xa21* gene for disease resistance isolated from pB822, *hph* gene for hygromycin resistance and *gus* gene of pCAMBIA1301 in T-DNA region (Baisakh *et al*., 2002). The gene for *gus* has intron in the 5' end of the coding sequence and is connected by 35S promoter of cauliflower mosaic virus (CaMV). The *Agrobacterium* strain EHA 101 (pTCL5) was grown overnight at 28ºC in YEP medium (An *et al*., 1988) containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin. It was then placed in shaker incubator at 100 rpm for 24-48 hours for bacterial multiplication.

Transformation and plant regeneration: At optimum bacterial density, three different dilutions (15, 7.5 and 3.75 ml) of bacterial culture were centrifuged at the speed of 3000 rpm for 15 minutes to obtain bacterial pellet which were then equally resuspended in 15 ml amino acid (AA) medium (Toriyama & Hinata, 1985) supplemented with 15 µl acetosyringone (As). Hypocotyl segments were dipped in bacterial suspension for 2-3 minutes and blotted dry on sterile filter paper. Explants were then placed on co-culture medium (1.5 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} Kinetin (CIM), 4 g l^{-1} gelrite and 100 µl As) with 1 ml of liquid medium (AA-As medium) spread over sterile filter paper placed on each Petri plate. Plates were sealed with parafilm and placed in dark at 28 ºC for 48 hours. After co-cultivation, the infected hypocotyls were washed for 2-3 minutes with liquid pre-selection media (CIM, 4 g l^{-1} gelrite, 500 mg l^{-1} cefotaxime) to control *Agrobacterium* attached to explants. After 7 days of pre-selection, hypocotyls (transformed) were placed on selection media (same as pre-selection media except that it contains 100 mg l^{-1} hygromycin) and left in growth room under 30 µmol m^{-2} s^{-1} cool fluorescent light in a 16 hr light/8 hr dark photoperiod at 25 ± 1ºC until callus growth occured. After 2-3 weeks of selection period, calli were transferred to the regeneration media (3.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA, 4 g l^{-1} gelrite, 500 mg l^{-1} cefotaxime and 100 mg l^{-1} hygromycin) for shoot regeneration. Regenerants were transferred to rooting medium (1.0 mg l^{-1} IBA, 4 g l^{-1} gelrite, 500 mg l^{-1} cefotaxime and 100 mg l^{-1} hygromycin) for full plant formation with extensive root system.

Histochemical GUS assay: Expression of GUS was assayed by incubating hypocotyl segments in X-gluc solution containing 50 mM Sodium phosphate buffer (pH 7.0), 1 mg X-gluc (5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide) in 1 ml 20 % methanol and 0.5 percent Triton X-100 (Jefferson, 1987). The reaction mixture was incubated overnight at 37 ºC after mild vacuum. For chlorophyll removal, plant material was soaked in 70 % ethanol and the blue staining was scored visually.

Statistical analysis: The experiment was completely randomized with three replicates and the data were analyzed using one way ANOVA in MSTAT program. Treatment means were evaluated at the $p < 0.05$ level of significance using least significant difference test. Data are presented graphically as means± standard errors of the means.

Results and Discussion

Effect of bacterial dilutions on transformation efficiency of chilli varieties; nepali and NARC-IV: Two main factors contributed to reproducibility of transformation protocol; (1) bacterial dilutions up to 1:4 (3.75 ml bacterial culture/15 ml AA medium) significantly enhanced transformation efficiency and (2) the response for transformation was highly dependent upon genotype.

Tissue necrosis of explants: Taking into account the mean number of hypocotyl showing tissue necrosis after 48 hrs exposure of co-cultivation (Fig. 2a), maximum number of explants survived at low bacterial concentration (117- 122.6) as compared to higher densities (81.02-106.6) in both varieties (Fig. 1a). Our results are in agreement to those obtained by Richter & Ronald (2000) that increased explant survival frequency at optimum bacterial density could be attributed to recognition of specific signal molecules from the invading pathogen which facilitated the process of T-DNA transfer in explants whereas, high bacterial inoculum resulted into rapid tissue necrosis (Fig. 2b) and cell death due to hypersensitive response characterized by a rapid, cell death around the infection site (Hansen, 2000). Severe necrosis of cauliflower hypocotyl explants was found by Chakrabarty *et al*., (2002) after exposure to undiluted culture of *Agrobacterium* (OD_{600} = 0.5) whereas necrosis was reduced to greater extent by exposure to diluted culture (1:10 and 1:20 dilution). Das *et al*., (2002) observed high tissue necrosis on leaf disc of grapes after 48 hours of co-cultivation with undiluted culture.

Number of pre-selected hypocotyls: After 7 days of pre-selection (Fig. 2c), substantial improvement in mean number of live explants was observed (Fig. 1b) at low density (103.3-113.8) that could be attributed to reduced tissue necrosis resulting from optimum *Agrobacterium* dilution. However, presence of bacteria after pre-selection in those exposed initially to higher bacterial concentrations led to necrosis and decreased the number and active growth of explants (70.0-86.34). According to Goodman & Novacky (1994) necrotic explants caused accumulation of antimicrobial substances (phytoalexins) which inhibited the potential of *Agrobacterium* to colonize plant cells and transfer T-DNA at high concentrations. Furthermore, Kuta & Tripathi (2005) were supportive of the view that such necrotic cells may attract other opportunistic microorganism under *In vitro* conditions which could result in serious contamination of cultures. Explants that exhibited healthy growth were transferred to selection medium.

Hygromycin selection of calli: Fig. 1c & 2d revealed that optimum bacterial concentration facilitated growth with subsequent selection of calli (72.67-87.32 calli) on selection medium (hygromycin) which varied significantly for both varieties. Better performance of calli on hygromycin indicated that living tissues were able to express gene resistance to hygromycin (Delis *et al.,* 2005). Presence of *Agrobacterium* at all the stages required frequent transfer to fresh medium. Poor response for calli selection with subsequent reduction in expression of hygromycin resistance gene in both varieties (44.32-72.31 calli) might be due to presence of necrotic regions and dead cells in the calli (Arillaga *et al*., 2001) when subjected initially to higher levels of concentrations.

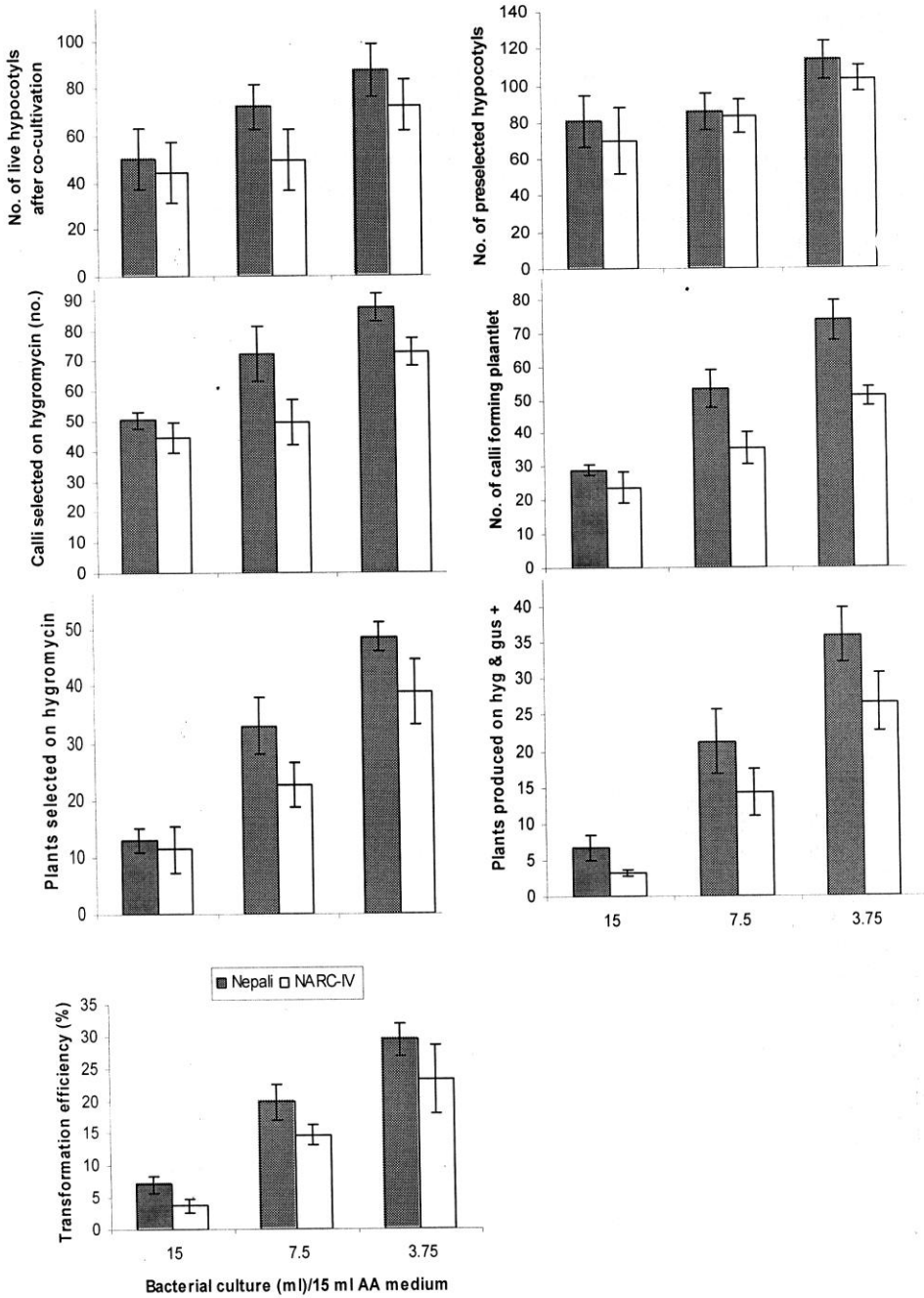

Fig. 1. Effect of different bacterial dilutions (ml of bacterial culture for pellet) on the number of live hypocotyls after co-cultivation, (a) No. of pre-selected hypocotyls, (b) calli selected on hygromycin, (c) No. of calli forming plantlet, (d) Plants produced on hygromycin, (e) Plant produced on Hyg & *gus* + (f) and % transformation efficiency (T. E) (g).

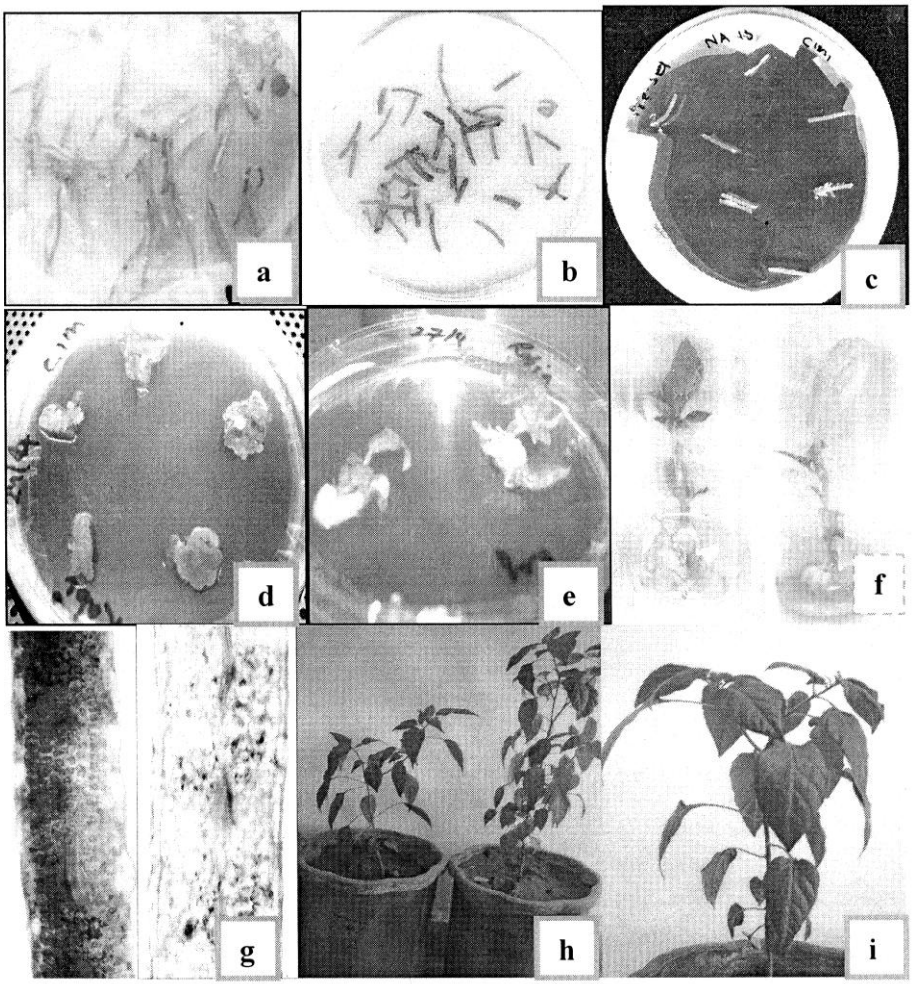

Fig. 2. Systematic representation of transformation protocols followed for Chilli varieties; co-cultivation of hypocotyls (a), Necrosis of explants following co-cultivation (b), Pre-selection (c) Selection of calli on hygromycin (d), Plant production on hygromycin (e), shoot elongation on medium containing 2 mg l^{-1} GA_3 (f), - ive & + ive GUS assay (g) and Acclimatization of Nepali (h) and NARC-IV (i) transgenic plants.

Plant formation from calli: Calli selected on hygromycin were transferred selection medium. Maximum production of plantlet in both varieties (51.0-73.67 calli) occurred for those treated initially with low bacterial density (Fig. 2e). Improved proliferation in such tissues might be supported by differentiation of cortex cells and vascular bundles while high density proved deleterious to calli proliferation (23.67-53.33) (Fig. 1d). Reduced recovery of transgenic cell clones with regards to regeneration might be related to inhibition of transgenic cells growth that is embedded in such necrotic tissues. Similarly, Delis *et al*., (2005) noted that increased necrosis of explants might result in death of explant cortex cells and vascular bundles. Moreover, hypertrophy of the cortex cells was responsible for the

tissue degeneration and subsequent reduced regeneration. In former studies, undiluted cultures (A_{600} = 0.7–1.0) adversely affected the growth and regeneration of callus during *Agrobacterium*-mediated transformation (Kumria *et al*., 2001).

Transgenic plant production: Calli showing plantlet formation were transferred to medium containing 2 mg l^{-1} of GA_3 (Fig. 2f). Moreover the trend set forth by the previous characters has been maintained here (Fig. 1e). Transgenic cell division and differentiation followed by shoot formation was maximum (39.0-48.67 plants) in both varieties on low bacterial inoculum. Hammond-Kosack & Jones (1996) demonstrated that effective expression and better shoot regeneration on medium containing hygromycin could be achieved only in living tissue as they possessed the ability to recognize invading pathogen thereby activating signal transduction which prevented colonization of bacteria. The active release of chemical signals which induces the *vir* genes in *Agrobacterium* occurs only in living tissues but not in dead necrotic tissues (Shaw *et al*., 1991). Reduced recovery of plants (11.0-33.03) in those pre-treated with high bacterial concentrations led to cellular disorganization in calli with subsequent reduction in shoot regeneration (Delis *et al*., 2005).

GUS expression and transformation efficiency (%): Mean number of plants expressing *gus* gene (26.67-36.0 plants) and transformation efficiency (23.25-29.47 %) varied significantly at low concentrations in both varieties. Substantial improvement in *gus* gene expression and transgenic plant production was only possible in active growing tissues not in necrotic tissues as seen in higher level concentrations (Fig. 1f & g). Repression of *gus* expression (3.30-21.0 plants) and reduced transformation efficiency (3.77-19.83 %) in plants might be due to physiological alterations that affected the delicate process of gene regulation which is induced in early culture stages. Similarly, the reactive oxygen species (hydroxyl radicals) produced during pathogen-induced oxidative burst in necrotic tissues could be toxic enough to directly kill the attacking *Agrobacterium* thus preventing transfer of T-DNA to plant cells (Wojtaszek, 1997). However, reduced recognition in these tissues could not establish a link between *Agrobacterium* and necrotic plant tissues resulting in death of cells where T-DNA was to be transferred. This in turn led to drastic reduction in transformation efficiency (Finnegan & McElroy, 1994). Nepali variety consistently remained responsive to transformation protocols as compared to NARC-IV variety. It clearly showed that plant cells can be highly susceptible or non-susceptible depending on the presence of competent cells, genotype of host plants and the strain of *Agrobacterium* (Jordan & Hobbs, 1994). Similarly, high transformation efficiency with reduced contamination and tissue necrosis in chilli varieties was achieved with dilution of bacterial culture up to 3.75 ml per 15 ml AA medium (1:4).

Histochemical GUS assay: Histochemical localization of GUS expression (Fig. 2*g*) driven by 35S-*gus*-intron construct in both varieties was high at low density (3.75 ml) which ensures that the GUS coloration arises from the transformed plant tissue and not from contaminating bacteria (Vancanneyt *et al*., 1990), while, in case of high bacterial concentration (7.5 and 15 ml of bacterial culture) reduced GUS expression occurred which showed that either gene is not transferred due to necrosis at such high densities or either it is non-functional due to transfer of plant origin of replication (Grierson, 1991).

Acclimatization: Well developed shoots were excised from callus and shifted to MS media for rooting. Root development occurred within 2 weeks and plantlets with fully expanded leaves and strong roots were transferred to pot containing soil and peat (1:1). Pots were covered with white polythene bag and placed in greenhouse with 95 % relative humidity. Plants were exposed to light intermittently until showed normal growth. After acclimatization of 2 wks in the greenhouse the plants of Nepali (Fig. 2h) and NARC-IV (Fig. 2i) were transferred to field for further development. The survival rate of plants in field was 25 % (data not shown).

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