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High Frequency Regeneration and *Agrobacterium tumefaciens*-mediated Transformation of Broccoli (*Brassica oleracea* var. *italica*)

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Summary

An efficient protocol for high frequency regeneration and *Agrobacterium tumefacies* mediated transformation of Broccoli *(Brassica oleracea* var. *italica)* has been developed. Hypocotyl and leaf segment explants were transferred on to MS medium supplemented with different concentrations and combinations of IAA (0–0.57 μM) and Kinetin/BAP/Zeatin (8.9–22.2 μM). Hypocotyl explants showed the highest regeneration potential. Maximum number of shoots (16 per explant) were obtained on MS medium supplemented with 0.57 μM IAA and 22.2 μM BAP. The concentrations of extracellular calcium, nitrate and ammonium nitrogen in the medium had a marked effect on regeneration. The optimal media for maximum shoot proliferation (30 per explant) contained 3 mM $CaCl_2$, 1900 mg l^{-1} $KN0_3$, 825 mg l^{-1} $(NH_4)_2S0_4$, 22.2 μM BAP and 0.57 μM IAA. Rhizogenesis from isolated shoots was obtained on B_5 medium supplemented with 2.85 μM IAA. The high frequency regeneration system served as an excellent tool to establish an efficient transformation method of broccoli. Hypocotyl explants were co-cultivated with the *Agrobacterium tumefaciens* strain LBA 4404, containing a binary vector with the neomycin phosphotransferase (*nptii*) gene and a β-glucuronidase (*gus*) gene, each under the control of a separate CaMV35S promoter.

Primary selection of transformed explants was performed on high kanamycin (100 mg l^{-1}) containing medium for 10 days and regeneration was achieved on the optimised regeneration medium containing lower concentration (50 mg l^{-1}) of kanamycin. The frequency of transgenic plants was calculated on the basis of GUS activity detected by histochemical X-gluc test. The effect of pre-culture and growth of bacterial culture was studied. Explants precultured for 2-days prior to inoculation with *A. tumefaciens* resulted in improved transformation frequency. Bacterial cultures grown to the optical density of 2.0 resulted in the highest transformation rate (60 %). Evidence for integration of *nptii* gene was obtained by the PCR. All transgenic plants grew normally. The present study demonstrates the improved high frequency regeneration and transformation of *B. oleracea* var. *italica.*

Key words. *Brassica oleracea* var. *italica* – regeneration – GUS – transformation

Zusammenfassung

Hochfrequente Regeneration nach Transformation mit *Agrobacterium tumefacies* bei Brokkoli *(Brassica oleracea* var. *italica)* Es wurde für Brokkoli *(Brassica oleracea* var. *italica)* ein effizientes Protokoll für die hochfrequente Regeneration nach Transformation mit *Agrobacterium tumefacies* entwickelt. Hypokotyl- und Blattsegment-Explante wurden auf MS-Medium mit unterschiedlichen Konzentrationen und Kombinationen von IAA (0–0.57 μM) und Kinetin/BAP/Zeatin (8.9–22.2 μM) kultiviert. Hypokotyl-Explante hatten das höchste Regenerationsvermögen. Die meisten Sprosse (16 pro Explant) wurden auf MS-Medium mit 0.57 μM IAA und 22.2 μM BAP gebildet. Die Konzentration von extrazellulärem Calcium, Nitrat- und Ammoniumstickstoff im Medium hatte eine deutliche Wirkung auf die Regeneration. Das optimale Medium für die maximale Sprossproliferation (30 pro Explant) enthielt 3 mM $CaCl_2$, 1900 mg l^{-1} $KN0_3$, 825 mg l^{-1} $(NH_4)_2S0_4$, 22.2 μM BAP und 0.57 μM IAA. Die Bewurzelung der Einzelsprosse erfolgte auf B_5-Medium mit 2.85 μM IAA. Das hochfrequente Regenerationssystem diente als hervorragendes Werkzeug zur Etablierung einer effizienten Brokkoli-Transformationsmethode. Hypokotyl-Explante wurden zusammen mit dem *Agrobacterium tumefaciens*-Stamm LBA 4404 kultiviert, der einen binären Vektor mit den jeweils von einem separaten CaMV35S-Promoter kontrollierten Genen für die Neomycin-Phosphotransferase (*nptII*) und die β-Glucuronidase (*gus*) enthielt.

Die erste Selektion der transformierten Explante erfolgte über zehn Tage auf Medien mit hoher (100 mg l^{-1}) und die Regeneration auf optimiertem Regenerationsmedium mit geringerer Kanamycinkonzentration (50 mg l^{-1}). Die Häufigkeit der transgenen Pflanzen wurde auf der Basis der GUS-Aktivität mittels histochemischem X-gluc-Test nachgewiesen. Die Wirkung einer Vorkultur und das Wachstum der Bakterienkultur wurde untersucht. Eine zweitägige Vorkultur vor der Inokulation mit *A. tumefaciens* führte zu einer verbesserten Transformationsrate. Bakterienkulturen, die bis zu einer optischen Dichte von 2.0 gewachsen waren, führten zur höchsten Transformationsrate (60 %). Der Nachweis der Integration des *nptii*-Gens erfolgte durch PCR. Alle transgenen Pflanzen wuchsen normal. Die vorliegende Untersuchung zeigt die verbesserte hochfrequente Regeneration und Transformation von *Brassica oleracea* var. *italica.*

Introduction

Broccoli *(Brassica oleracea* var. *italica)* is an important vegetable and is recognized as one of the most nutritious crops, especially in calcium, antioxidants, vitamin A, vitamin K, β-carotene, riboflavin and iron content (VALLEJO et al. 2003; ABDEL-WAHHAB and ALY 2003). The anti-cancer properties of broccoli are contributed by sulforaphane glucosinolate (KECK et al. 2003), quinone reductase glutathione S-transferase (ZHANG et al. 1992; FAHEY et al. 1997) and high selenium content (FINLEY et al. 2001; FINLEY 2003). The crop yield is drastically affected by various fungal pathogens such as *Alternaria brassicae, Fusarium oxysporum, Rhizoctonia solani, Erysiphe polygoni* and most important *Peronospora parasitica* causing downy mildew. Thus, there is an urgent need for crop improvement.

Classical breeding techniques are insufficient for the genetic improvement. Plant breeders in the past several decades have used inter-specific sexual hybridisation to transfer genes between species. Sexual incompatibility barriers severely limit the possibilities for gene transfer between species, although some of the *Brassica* can be easily crossed sexually and through somatic hybridisation (PUDDEPHAT et al. 1996). Thus, genetic engineering can be used to add specific characteristics to existing cultivars. However, a pre-requisite for transferring genes into plants is the availability of efficient regeneration and transformation methods. Plant tissue culture techniques provide an excellent opportunity for crop improvement. Cloned populations are of interest because the self-incompatibility and biennial life cycle of many *B. oleracea* crops, including broccoli, can make the recovery of sufficient seeds for large scale studies slow and difficult. Micropropagation of *B. oleracea* var. *italica* has been reported (ANDERSON and CARSTENS 1977; CHRISTEY and EARLE 1991; CHOPRA and NARASIMHULU 1991; MUANGKAEWNGAM and TECHATO 1992; FARNHAM and NELSON 1993; MSIKITA et al. 1997). Various varieties of *B. oleracea* have been transformed via *Agrobacterium rhizogenes* (HOSOKI et al. 1989, 1991; BOULTER et al. 1990; CHRISTEY and SINCLAIR 1992; CHRISTEY et al. 1997; HENZI et al. 1999, 2000). Transformation of *B. oleraca* via *Agrobacterium tumefaciens* have been reported (DE BLOCK et al. 1989; METZ et al. 1995a, b; CHENG et al. 2001a) and transgenic broccoli has been subjected to field trials (WATERER et al. 2000). Recently, CAO and EARLE (2003) reported high frequency shoot formation from leaf explants of transgenic broccoli plants expressing two *bt* endotoxin genes (*cry1C* and *cry1AC*). Among the various factors affecting transformation efficiency and recovery of transgenic plants, an improved high frequency regeneration technique is most important.

Therefore, the objectives of the present study were to improve and establish an efficient protocol for high frequency regeneration and *A. tumefaciens* mediated transformation system in *B. oleracea* var. *italica.*

Materials and Methods

Plant materials

Broccoli *(B. oleracea* var. *italica* cultivar 'Windsor' (Syngenta Seeds Inc., Boisc) seeds were surface sterilized by using 0.1% mercuric chloride for three minutes followed by five rinses with sterile distilled water. Seeds were germinated aseptically in Magenta boxes containing 50 ml Murashige and Skoog's (MS) basal media (MURASHIGE and SKOOG 1962) with 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. Hypocotyls (15 mm) and leaf segment (5x5 mm) explants from 15-day old seedlings were used for regeneration experiments.

Optimization of shoot induction and multiplication

Hypocotyl and leaf segment explants were transferred on to MS medium supplemented with different concentrations and combinations of indoleacetic acid (IAA; 0 and 0.57 μM) and cytokinins (8.9, 13.3 and 22.2 μM) viz., kinetin, 6-benzylaminopurine (BAP) and zeatin. Each culture flask (250 ml, 'Borosil') contained 50 ml medium and 2 explants. The effect of nutritional constitution of media on *in vitro* regeneration of broccoli was analysed by using B_5 (GAMBORG et al. 1968) and Nitsch (NITSCH and NITSCH 1969) and MS full / half-salts medium each containing 0.57 μM IAA and 22.2 μM BAP. The concentration of $CaCl_2.2H_2O$ in MS media was varied from 0–6 mM. The source of nitrate- and ammonium-nitrogen in MS medium containing 3 mM calcium chloride, 0.57 μM IAA and 22.2 μM BAP was manipulated by using different concentrations and combinations of KNO_3 (1900 and 3800 mg l^{-1}) and $(NH_4)_2SO_4$ (206.2–1650 mg l^{-1}), respectively. Thus, the total nitrogen content was varied from 335.2 to 1103.5 mg l^{-1}N.

Rhizogenesis

In vitro derived shoots bearing 4–6 leaves were transferred onto MS half-salts or B_5 medium containing charcoal (2 g l^{-1}) and different concentrations (0.3–2.85 μM) of indoleacetic acid (IAA) or indolebutyric acid (IBA). After 8 weeks of growth plantlets were planted in plastic pots containing a sterilized soil: vermiculite mixture (3:1,V/V). To maintain high ambient humidity the pots were covered with transparent polyethylene bags with pores. Plants were irrigated (25 ml pot^{-1}, tap water) on alternate days. After one-month growth, plants were transferred to the glasshouse.

Bacterial strains

A. tumefacies strain LBA 4404 containing a binary vector, pGA 472 with the GUS as the reporter gene, *nptII* gene as plant selective marker and pTi Bo542 as the helper plasmid (AN et al. 1987) was used for transformation. *gus* and *nptII* genes were under the control of a separate CaMV35S promoter. For inoculation, the bacteria were cultured overnight at 28 °C in yeast extract media supplemented with 10 mg l^{-1} rifamycin, 50 mg l^{-1} streptomycin and 50 mg l^{-1} kanamycin. Bacteria were grown for different times (optical density A_{600}= 0.1 to 0.6) in liquid yeast extract medium, centrifuged at 4000 rpm, re-dissolved in MS hormone-free media and used for inoculation.

Transformation

Hypocotyl explants were used for transformation. For co-cultivation, hypocotyls were immersed in the bacte-

rial suspension for 5 minutes, blotted dry and transferred on to modified MS (MMS) medium (MS medium devoid of NH_4N0_3 and containing 825 mg l^{-1} $(NH_4)_2S0_4$) with 22.2 µM BAP and 0.57 µM IAA. Co-cultivation was conducted for 2days in the dark at 25 °C. In another experiment, explants were pre-cultured for 2 days on modified MMS medium supplemented with 0.57 µM IAA, 22.2 µM BAP prior to the inoculation. Control explants were treated similarly, except that they were incubated in sterile yeast extract medium. After co-cultivation, explants were transferred to MMS medium supplemented with 0.57 µM IAA, 22.2 µM BAP, 500 mg l^{-1} cefotaxime for elimination of the *Agrobacterium* and 100 mg l^{-1} kanamycin for selection. The concentration of kanamycin was reduced to 50 mg l^{-1} after 10 days of growth.

Histochemical GUS assay

Histochemical GUS assays were conducted on the explants 30 days after co-cultivation using the method described by JEFFERSON (1987). Briefly, explants were fixed for 45 min in 0.3 % formaldehyde, 10 mM MES (pH 5.6) and 0.3 M Mannitol, incubated in 1 mg ml^{-1} X-gluc (in phosphate buffer, pH 7.0 containing 50 mM potassium ferricyanide, 50 mM potassium ferrocyanide and 20 % methanol) at 37 °C overnight. Tissues were cleared with a mixture of formaldehyde, acetic acid, ethanol, and water (2:10:10:7) overnight. The number of blue shoot buds per explants was scored.

DNA isolation and PCR screening

Approximately 200 mg of leaf tissue of transgenic plants was grounded to a powder in liquid nitrogen and total DNA was extracted by modified CTAB method (POREBSKI et al. 1997). Detection of neomycin phosphotransferase (*nptii*) gene in transgenic plants was performed using polymerase chain reaction (PCR) with primers 5'-TCAAGAAGGCGATAGAAGGCGATCG-3' and 3'-CTGTCATCTCACCTGGCTCCTGCCG-5', which were designed to amplify approximately 490 bp DNA fragment of the *nptii* gene. Each 25µl reaction volume contained 50 ng DNA, 0.20 mM each of dNTPs, 15 ng of each primer, 2.5 mM $MgCl_2$, 2.5 µl 10X specific reaction buffer, and 0.5 U *Taq* DNA polymerase (Qiagen). DNA samples were denatured at 94 °C for 5 min and amplified during 40 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Amplified PCR products were electrophorised in 1.5 % (w/v) agarose gel.

Autoclaving, culture conditions and statistical analysis

The pH of the medium was adjusted to 5.8 with 0.5 N HCI or NaOH before autoclaving at 121 °C for 15 min. Cultures were incubated at 26±0.5 °C under white fluorescent light (Philips cool TL 36W/54) with a total irradiance of 36 µmol m^{-2} s^{-1} for 16 hours photoperiod and 60±5 % relative humidity. Experiments were conducted using 8 replicates per treatment and data was analysed by using 2x3x3 factorial RBD-ANOVA, 2x4x4 factorial RBD-ANOVA, Single factor ANOVA, and 2x4 factorial CRD-ANOVA analysis.

Results

Effect of plant growth regulators on regeneration

Leaf segment (5x5 mm) and hypocotyl (15 mm) explants were tested for their regeneration ability. These explants when transferred on to MS medium supplemented with different concentrations and combinations of auxins (0 and 0.57 µM) and cytokinins (8.9, 13.3 and 22.2 µM) viz., kinetin, BAP and zeatin, produced varied number of shoots. Leaf segment explants regenerated the maximum number of shoots (4 per explant) on MS medium containing 22.2 µM BAP (Table 1) and shoot initiation was observed only after 20 days of incubation at the best. Presence of IAA in the media significantly inhibited shoot initiation and proliferation from leaf explants. In contrast, hypocotyl explants produced the maximum number of shoots (16 per explant) on MS medium containing 22.2 µM BAP and 0.57 µM IAA (Table 1; Fig. 1, 2). Initiation of shoot buds from hypocotyl explants was observed within 10–15 days of incubation. Among the various cytokinins tested, BAP was found to be significantly most effective for shoot induction and proliferation. Thus, the present investigation showed that hypocotyl explants were best suitable for regeneration.

Effect of media formulations, extra-cellular calcium and nitrogen on the regeneration

In the attempt to further improve the regeneration ability of *B. oleracea* var. *italica* most of the media factors tested did not enhance regeneration. Various media formulations were compared to optimise regeneration. MS medium was found to be optimal for regeneration (Table 2). Low salts media significantly reduced shoot regeneration from hypocotyl explants and Nitsch media drastically reduced shoot proliferation. Extracellular calcium also played an important role in regeneration. Maximum numbers of shoots (20 per explant) were obtained on the original MS medium containing 3 mM $CaCl_2$. Depletion of calcium in the media, drastically inhibited shoots proliferation (Table 3).

The regeneration potential of *B. oleracea* var. *italica* was further improved by manipulating nitrate- and ammonium-nitrogen in the media. Concentration of KNO_3 higher than the original MS medium, significantly reduced shoot proliferation (Table 4). The NH_4N0_3 of the original MS medium described by MURASHIGE and SKOOG (1962) was replaced with $(NH_4)_2S0_4$ in order to manipulate the ratio of nitrate:ammonium nitrogen. Maximum number of shoots (30 per explant) were obtained *in vitro* from hypocotyl explants on MS medium containing 1900 mg 1^{-1} KNO_3 and 825 mg 1^{-1} $(NH_4)_2S0_4$ indicating that lower concentration of ammonium nitrogen contributed by $(NH_4)_2S0_4$ significantly improved shoot regeneration. The optimal medium for rapid regeneration in *B. oleracia* var. *italica* was MS medium containing 3 mM $CaCl_2$, 1900 mg 1^{-1} KNO_3, 825 mg 1^{-1} $(NH_4)_2S0_4$, 22.2 µM BAP and 0.57 µM IAA.

Maintenance of cultures

Regenerating cultures were maintained and multiplied by longitudinally splitting shoot clusters and sub-cul-

Table 1. Effect of different concentrations and combinations of IAA and various cytokinins viz., kinetin, BAP and zeatin, on shoot formation from leaf segment explants and hypocotyl explants.

IAA (μM)	Cytokinin (μM)		Leaf segment explant				Hypocotyl explant			
			Response (%)	Initiation time (days)	*No. of shoots / explant ± s.d	Shoot length (cm ± s.d)	Response (%)	Initiation time (days)	*No. of shoots / explant ± s.d	Shoot length (cm ± s.d)
0	Kinetin	8.9	75	25	1.2 ± 0.5	2.5 ± 0.7	80	12	2.4 ± 1.2	1.7 ± 1.5
		13.3	100	26	2.3 ± 0.5	2.1 ± 1.0	80	12	6.3 ± 1.6	4.2 ± 1.2
		22.2	80	26	3.0 ± 0.8	2.0 ± 0.9	100	12	9.6 ± 1.3	1.2 ± 1.0
	BAP	8.9	80	23	1.8 ± 0.5	2.1 ± 1.0	80	12	6.6 ± 1.7	3.1 ± 1.1
		13.3	100	24	2.5 ± 0.6	2.1 ± 0.5	100	10	10.0 ± 2.0	4.0 ± 0.9
		22.2	100	25	4.0 ± 0.8	2.2 ± 0.9	100	10	14.8 ± 1.8	4.6 ± 0.9
	Zeatin	8.9	50	20	1.2 ± 0.5	1.0 ± 1.1	75	14	7.8 ± 1.3	3.5 ± 1.3
		13.3	75	20	2.8 ± 0.5	2.3 ± 0.8	100	12	10.5 ± 2.0	4.3 ± 1.4
		22.2	100	20	1.5 ± 0.7	3.0 ± 1.2	100	10	11.2 ± 1.4	2.1 ± 1.0
0.57	Kinetin	8.9	75	25	1.0 ± 0.8	2.7 ± 1.0	80	14	3.1 ± 1.1	2.7 ± 1.0
		13.3	80	26	1.5 ± 0.6	3.6 ± 1.2	50	10	6.7 ± 1.1	3.6 ± 0.7
		22.2	50	29	2.0 ± 0.9	2.9 ± 0.9	75	10	10.6 ± 1.3	3.9 ± 1.2
	BAP	8.9	50	29	1.0 ± 0.7	2.2 ± 0.5	80	12	11.6 ± 1.5	2.2 ± 1.1
		13.3	50	30	2.5 ± 0.6	3.3 ± 1.0	100	12	12.6 ± 1.7	3.3 ± 1.2
		22.2	50	30	3.0 ± 0.8	5.1 ± 0.7	100	10	16.1 ± 1.5	5.1 ± 1.0
	Zeatin	8.9	75	25	1.2 ± 0.5	2.1 ± 1.0	100	15	6.3 ± 1.6	2.1 ± 0.8
		13.3	50	20	2.3 ± 0.5	3.6 ± 1.2	100	15	10.7 ± 2.6	3.2 ± 1.3
		22.2	50	25	1.5 ± 0.7	4.0 ± 0.9	100	10	11.1 ± 1.6	4.0 ± 0.9

*****IAA: Significant at 5 %, SE = 0.21, $CD_{5\%}$ = 0.43
Cytokinin:Significant at 1 %, SE = 0.45, $CD_{1\%}$ = 1.23
IAA X Cytokinin: Significant at 20 %, SE = 0.64, $CD_{20\%}$ = 0.83

*****IAA: Significant at 2 %, SE = 0.36, $CD_{2\%}$ = 0.88
Cytokinin: Significant at 10 %, SE = 0.78, $CD_{10\%}$ = 2.14
IAA X Cytokinin: Significant at 1 %, SE = 1.10, $CD_{1\%}$ = 1.86

turing on the induction medium. No decline in regeneration potential was observed. However, sub-culturing in intervals of 20 days resulted in fast growing healthy miniature shoots, whereas prolonged subculture periods of 30 days generated yellowing of shoots.

Rhizogenesis

Miniature shoots obtained on the regeneration media described above were rooted on B_5 and MS half-salts medium supplemented with different concentrations (0.30–2.85 μM) of IAA or IBA. Maximum number of roots was obtained on B_5 media supplemented with 2.85 μM IAA (Table 5; Fig. 3). An average of over 8 roots per plant were obtained on this medium. Initiation of root was observed in 4 days of incubation on B_5 medium containing 2.85 μM IAA. The effect of media composition and auxin concentration on rhizogenesis was significant. More than 90 % of the plants rooted on these media survived the transfer to soil and showed no morphological abnormalities.

Transformation with Agrobacterium tumefaciens.

An efficient protocol for high frequency *A. tumefaciens*-mediated transformation of broccoli has been established. Hypocotyl explants were used in the present study. Following inoculation, explants were primarily selected for possible successful transformation on the optimal regeneration medium (described above) containing 100 mg l^{-1} kanamycin. After 10 days of growth on this selection medium, the concentration of kanamycin was reduced to half. Untransformed explants (negative control) turned brown or white on the selection medium (Fig. 4). The transformation frequency was calculated performing histochemical X-gluc test. Transgenic shoot buds showed dark blue spots indicating constitutive GUS expression (Fig. 5). Explants pre-cultured for 2 days on the optimised regeneration medium prior to inoculation resulted in improved transformation frequency (60%), which was about 2 times higher than explants infected without pre-culture. Growth of the bacterial cultures also played an important role in transformation. Over-grown bacterial cultures ($OD_{600nm} \geq 0.4$) drastically reduced transformation frequency. Maximum transformation frequency (60%) was attained by pre-culturing hypocotyls explants for 2 days on MMS medium supplemented with 22.2 μM BAP and 0.57 μM IAA prior to infection with bacterial cultures grown to $OD_{600\ nm}$ = 0.2. When the OD was ≥ 0.5, transformation frequency was reduced drastically to 10%. Transgenic shoot buds when sub-cultured on the same medium produced well-developed shoots, which were rooted as described above. Transgenic plants were successfully transferred to soil and the growth was comparable to the control. The PCR analysis of transgenic plants confirmed integration of the *nptII* gene indicating successful transforma-



Fig. 1. Shoot induction from 15-days old hypocotyl explants of *B. oleracea* var. *italica*.



Fig. 2. Multiple shoot formation from hypocotyl explants on MS medium supplemented with 3 mM $CaCl_2$, 263 mg Nl^{-1} KNO_3, 288.7 mg N $l^{-1}(NH_4)_2SO_4$, 22.2 µM BAP and 0.57 µM IAA.

Table 2. Effect of different media compositions on shoot formation from hypocotyl explants.

Media	Response (%)	Initiation time (days)	*No. of shoots / explant ± s.d	Shoot length (cm ± s.d)
MS half-salts	100	11	13.8 ± 1.7	4.7 ± 0.9
MS	100	9	16.8 ± 2.1	5.2 ± 1.0
B_5	75	8	13.5 ± 1.8	3.3 ± 1.2
Nitsch	80	12	2.6 ± 1.4	2.0 ± 1.0

*Significant at 1 %, SE = 0.06, $CD_{1\%}$ = 0.17

Table 3. Effect of different concentrations of extracellular calcium on shoot formation from on hypocotyl explants.

[$CaCl_2.2H_2O$] (mM)	Response (%)	Initiation time (days)	*No. of shoots / explant ± s.d	Shoot length (cm ± s.d)
0	50	10	7.6 ± 1.2	2.8 ± 1.5
1	80	7	10.0 ± 1.3	3.5 ± 1.3
2	100	12	12.1 ± 1.2	3.3 ± 0.9
3	100	9	20.3 ± 2.5	6.2 ± 1.4
4	100	9	15.7 ± 1.6	5.0 ± 1.6
5	100	6	13.3 ± 2.0	4.0 ± 0.9
6	90	10	12.2 ± 1.1	3.4 ± 1.1

tion. Approximately 500 bp band representing DNA fragment of the *nptII* gene was observed (Fig. 6).

Discussion

This study describes the optimisation of regeneration and *Agrobacterium tumefaciens*-mediated transformation of broccoli, an important vegetable crop. Hypocotyl and leaf segment explants were used to standardize regeneration. Our results showed that hypocotyl explants were best suitable for *in vitro* regeneration whereas regeneration from leaf explants was 4 times lower as compared to hypocotyls explants. *Brassica* leaf explants are generally known to be a difficult source for micropropagation (BHALLA and WEERD 1999; CHENG et al. 2001a).

Various explants viz., leaf, hypocotyls, petiole, and flowering stalk have been previously tested but the regeneration was low for applied applications (METZ et al. 1995a; CAO et al. 1999). Recently, CAO and EARLE (2003) reported improvement in regeneration by rapid subcultures, use of younger leaves and appropriate concentration of growth regulators. They concluded that the cells sharing same genetic blue print have acquired different regulatory programs to become competent for shoot induction, with leaves requiring an auxin and cytokinin to become competent for shoot induction.

In the present study, the effect of various plant growth regulators, media formulations, extracellular calcium, and nitrogen on regeneration was studied. Low salt media significantly reduced shoot proliferation. The source of nitrate- and ammonium-nitrogen contributed by KNO_3 and $(NH_4)_2S0_4$, respectively had a marked effect on regeneration of broccoli. This is supported by our previous reports indicating the role of nitrogen source in shoot proliferation and somatic embryogenesis in several plant species (SURI et al. 1998, 1999, 2000). Lower concentrations of ammonium nitrogen in the media resulted in forced regeneration. Maximum number of shoots (30 per explant) were obtained on modified MS (MMS) medium (MS medium containing 3 mM $CaCl_2$, 1900 mg l^{-1} KNO_3, 825 mg l^{-1} $(NH_4)_2S0_4$ with 22.2 µM BAP and 0.57 µM IAA.

The availability of an efficient protocol for high frequency forced regeneration is a pre-requisite for generating transgenic plants through transformations. We describe here an efficient protocol for *Agrobacterium tumefaciens*-mediated transformation of broccoli using hypocotyl explants. Various varieties of *B. oleracea* have been transformed via *A. rhizogenes* (HOSOKI et al. 1989;

Table 4. Effect of KNO_3 and $(NH_4)_2SO_4$ concentrations in MS medium supplemented with 3 mM $CaCl_2$, 22.2 µM BAP and 0.57 µM IAA on shoot proliferation from hypocotyls explants.

KNO_3		$(NH_4)_2SO_4$		Total nitrogen	Response	Initiation time	*No. of shoots /	Shoot length
mg l^{-1}	[mg l^{-1} N]	mg l^{-1}	[mg l^{-1} N]	(mgl^{-1}N)	(%)	(days)	explant ± s.d	(cm ± s.d)
1900	[263]	206.2	[72.17]	335.2	25	15	5.8 ± 1.2	1.1 ± 0.5
		412.5	[144.3]	407.3	75	10	18.2 ± 2.7	3.1 ± 1.1
		825.0	[288.7]	551.7	100	6	29.7 ± 2.2	6.4 ± 1.5
		1650.0	[577.5]	840.5	100	12	21.0 ± 2.6	5.1 ± 1.0
3800	[526]	206.2	[72.17]	598.2	20	20	4.5 ± 1.3	0.9 ± 0.4
		412.5	[144.3]	670.3	50	10	14.5 ± 2.0	3.5 ± 1.3
		825.0	[288.7]	814.7	75	8	16.0 ± 2.2	4.1 ± 0.9
		1650.0	[577.5]	1103.5	80	12	9.7 ± 1.7	2.7 ± 1.2

*****KNO_3: Significant at 1 %, SE = 0.84, $CD_{1\%}$ = 2.41
$(NH_4)_2SO_4$:Significant at 1 %, SE = 1.18, $CD_{1\%}$ = 3.39
KNO_3 X $(NH_4)_2SO_4$: Significant at 1 %, SE = 1.67, $CD_{1\%}$ = 4.80

BOULTER 1990; CHRISTEY and SINCLAIR 1992; CHRISTEY et al. 1997; HENZI et al. 2000) and *Agrobacterium tumefaciens* (DE BLOCK et al. 1989; METZ et al. 1995a, b). Broccoli has also been transformed with *bt* (METZ et al. 1995a; CAO et al. 2002) and isopenthenyltransferase (CHENG et al. 2001b) genes. HENZI et al. (2000) reported transformation rate of 70 % (*gus* gene) and 33 % (ACC oxidase gene) in broccoli. The present study describes the effect of pre-culture and growth of bacterial cultures on the efficiency of transformation. Explants pre-cultured for 2 days prior to inoculation resulted in high transformation frequency. MC HUGHEN et al. (1989) and METZ et al. (1995a) also reported similar results. We observed that inoculation of explants with low-density bacterial cultures was beneficial. The role of various other factors such as, arginine, mannopine and acetosyringone has been studied in affecting *A. rhizogenes*-mediated transformation in broccoli (HENZI et al. 2000).

The protocol for regeneration and *A. tumefaciens*-mediated transformation of broccoli in the present report is reproducible and might be useful for transforming broccoli with genes of agricultural importance.

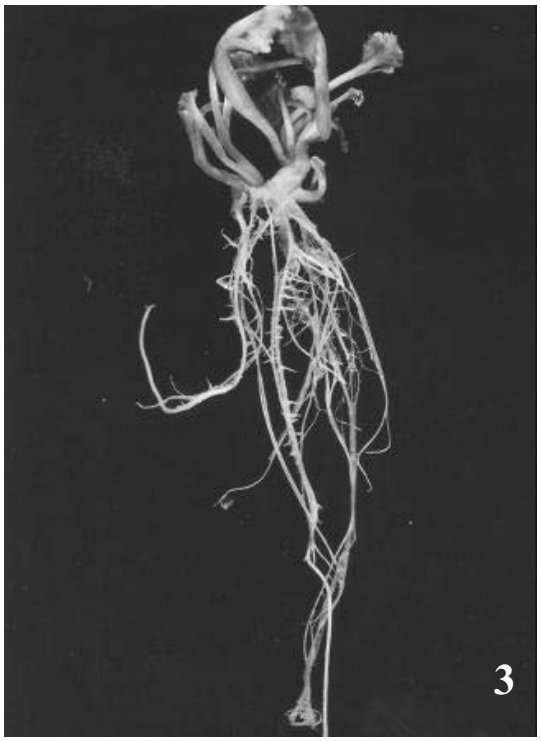


Fig. 3. A plantlet with multiple roots on B_5 medium containing 2.85 µM IAA.

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Table 5. Effect of different concentrations of IAA or IBA in MS half-salts and B_5 medium on rhizogenesis of *in vitro* generated shoots.

Auxins (μM)		MS half-salts media				B_5 media			
		Response (%)	Initiation time (days)	*No. of roots ± s.d	Root length (cm±s.d)	Response (%)	Initiation time (days)	* No. of roots ± s.d	Root length (cm ± s.d)
IAA	0.30	75	15	3.0 ± 1.0	4.7 ± 2.7	100	10	5.0 ± 0.8	7.7 ± 1.0
	0.55	100	10	3.5 ± 0.5	6.8 ± 0.8	100	8	5.5 ± 0.5	12.2 ± 1.7
	1.15	100	8	4.5 ± 0.7	8.5 ± 0.5	100	6	6.7 ± 0.7	16.0 ± 0.8
	2.85	100	8	4.6 ± 1.2	10.5 ± 1.2	100	4	8.2 ± 1.2	10.0 ± 1.8
IBA	0.30	50	12	2.6 ± 0.9	3.7 ± 0.9	75	5	3.8 ± 0.6	17.2 ± 1.7
	0.55	75	8	4.8 ± 0.7	7.0 ± 0.5	100	8	4.7 ± 1.2	13.0 ± 1.4
	1.15	100	10	4.2 ± 0.7	11.2 ± 1.4	100	10	7.1 ± 1.2	15.5 ± 1.3
	2.85	50	10	3.6 ± 0.5	7.0 ± 0.8	100	10	6.0 ± 1.1	7.2 ± 1.5

*Media: Significant at 1 %, SE = 0.66, $CD_{1\%}$ = 1.81
Auxin: Significant at 10 %, SE = 0.93, $CD_{10\%}$ = 1.58

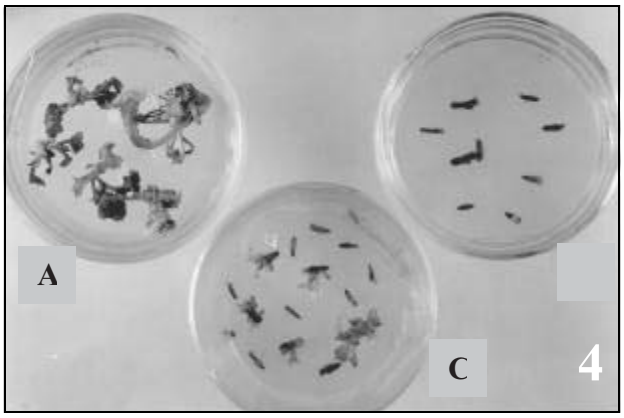


Fig. 4. Comparison of regeneration potential of positive (A) and negative (B) controls with transformed explants (C).

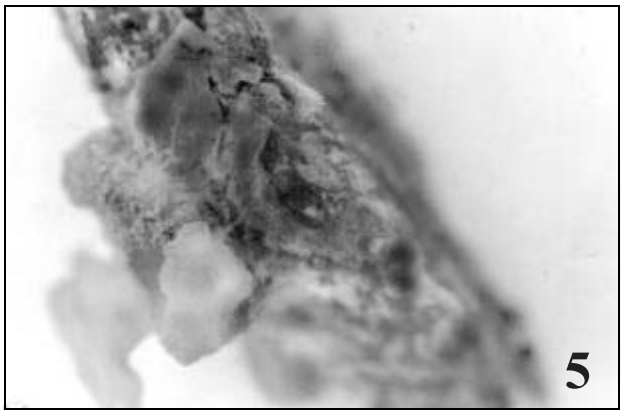


Fig. 5. Histochemical GUS assay of regenerating transgenic hypocotyls explant showing blue spots.

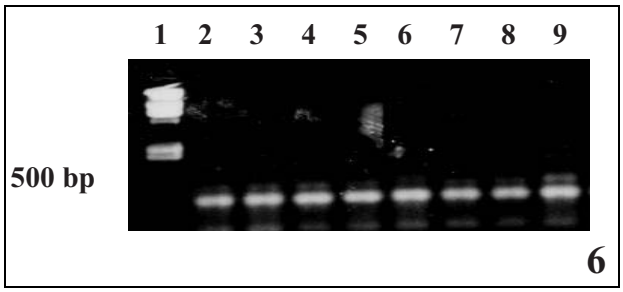


Fig. 6. Gel electrophoresis of PCR products amplified with NPTII primers. Lane 1: molecular weight marker 1 kb, transgenic broccoli; lane 2 to 9: showing ~490 bp amplified region of *nptii* gene.

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