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STRAIN AND VECTOR SPECIFICITY IN *AGROBACTERIUM* – BLACK PEPPER INTERACTION

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ABSTRACT

Two *Agrobacterium tumefaciens* binary vector strains LBA 4404 and EHA 105, both with pGA and a new co-integrate vector (PGV 2260- PGSFR 280) were used for transformation of black pepper. Cotyledons of 3-4 month old 'Karimunda' variety seedlings were the explant. Infected explants were grown in MS selection media containing 50 μg ml^{-1} kanamycin and 500 μg $^{-1}$ carbencillin in case of the binary vectors and 50 μg ml^{-1} kanamycin, 500 μg $^{-1}$ carbencillin and 250 μg ml^{-1} cefotaxime in case of the co- integrate vector. Twenty per cent primary transformants each could be obtained in case of the binary vectors whereas no appreciable 'transformation' could be obtained in case of the co- integrate vector. 'Transformation' was confirmed by phenotypic assay only.

INTRODUCTION

One of the most widely used system of genetically engineering crop plants is based on the Ti (tumour inducing) plasmid and Ri (root inducing) plasmids of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes,* respectively. In order for genetic modification using *Agrobacterium* to be successful, an effective transformation and regeneration system must be in place. Successful and effective transformation by *Agrobacterium* depends not only on the plant species, cultivar, explant source, but also on the bacterial strain, type of vector and transformation techniques (Davis *et. al.*, 1991). It is felt that in certain plant species co-integrate vectors are more efficient transformation vectors as compared to the binary vectors and transformation of plants like tomato is still better done with the cointegrate vectors. It has also been known for sometime that some wild type *Agrobacterium* strains are more virulent on certain dicotyledonous species than on others (Byrne *et. al.,* 1987) and this virulence is attributed to the efficiency of particular *vir* genes. The aim of the present work is to study the transformation differences among two binary vector strains and a co-integrate vector strain on black pepper.

MATERIALS AND METHODS

Bacterial strains: 1) Disarmed *Agrobacterium tumefaciens* binary vector strain LBA 4404 and EHA 105 containing pGA 472. EHA 105 is a supervirulent strain and

2) Disarmed *Agrobacterium tumerfaciens* co-integrate vector containing plasmid PGV 2260 - PGSFR 280.

Plant material: Cotyledon explants and primary leaves of 3-4 month old black pepper cultivar 'Karimunda' raised in sand filled basins were used.

Surface sterilization : Cotyledons and primary leaves were separated, washed thoroughly in running tap water and sterilized sequentially in: a) 70% ethanol for five minutes followed by thorough washing in sterilized water, b) 40% v/v sodium hypochlorite plus few drops of Tween 80 for five minutes followed by

thorough washing in sterilized water. c) 0.1% $HgCl_2$ for three minutes. Finally the explants were washed thoroughly in sterilized water, transferred to a sterile petriplate with filter paper and cut horizontally into segments of about 0.2 x 1.5 cm size after cutting off the edges.

Cultural conditions : The explants were cultured on a medium containing Murashige and Skoog salts and vitamins 40 gl^{-1} sucrose plus BAP 3 mgl^{-1}, NAA 2 mg l^{-1} and 2, 4-D 1 mg l^{-1}. The medium was adjusted to pH 7.0 before autoclaving and dispensed as 25 ml aliquots into 100 ml conical flasks. There were four flasks/treatments with 3-5 explants each to a single flask. All cultures were maintained at 25 $\pm 1^0$C under 16 h photoperiod.

Transformation: Both the binary vector strains carried the binar plasmid pGA 472 which contains a neomycin phosphotransferace (NPT-11) gene under the control of a nopaline synthase promoter. The co-integrate vector contained the plasmid PGV 2260 - PGSFR 280 formed by co-integrating PGSFR 280 carrying an NPT II gene with the resident plasmid PGV 2260. Bacterial cultures were grown for 18 hr in case of the co-integrate vector and 35 hr in case of the binary vector (reaching 0.7 to 1.0 D) in Ab minimal medium containing 2.5 µg ml^{-1} tetracycline (binary vectors) and 150 mg ml^{-1} streptomycin (co-integrate vector) at 30^0C with shaking. Preincubated explants (for two days) in drug free medium were submerged in the bacterial culture, with gentle shaking of the petriplates for 2-3 minutes, blotted dry on a sterile filter paper and co-cultivated on fresh medium (without drug) for another two days. Explants infected with the binary vector strains were then transferred to selection medium containing 50 µg ml^{-1} kanamycin (Kanamycin monosulfate) and 500 µg ml^{-1} carbencillin, while the explants infected with the co-integrate vector were transferred to selection medium containing 100 µg m l^{-1} kanamycin, 500 µg m l^{-1} carbencillin and 250 µg ml^{-1} cefotaxime. The explants were subcultured onto fresh selection medium periodically. Control (control-1) explants did not receive the infection and they were left on the same medium but without the antibiotics. Another set of uninfected control (control-2) was left on selection medium with inhibitory levels of the drugs.

Phenotypic assay for confirmation of transformation : In order to confirm the transformation of the tissues, 'transformed' and control calli were raised on higher concentrations of kanamycin (the selective marker). Kanamycin concentrations of 50, 100, 150, 200 and 300 µg ml^{-1} with 500 µg ml^{-1}carbercillin were used in the study. Two sets of flasks were kept in each case. Culture media and culture conditions remained the same described above. Growth and proliferation of calli were monitored.

RESULTS AND DISCUSSION

About 20% each callusing was observed in cotyledon explants of black pepper, 'Karimunda' infected with the *Agrobacterium* strain LBA 440 and EHA 105 and cultured on the respective selection media (Table I). There was no appreciable difference in the transformation frequency of the two binary vector strains. The cotyledon explants infected with the co-integrate vector did no callus. But slight swelling of the cotyledon segments were observed which subsequently died. All the infected explants from the primary leaves of black pepper remained alive even after three months of culturing in selection medium but no appreciable callus development could be observed in this material. However, slight swelling and building of the tissues were evident. Control explants on selection medium (control -1) in all cases had about 90% callusing. The control calli which was

Table I. **Frequency of callusing from black pepper explants infected with *Agrobacterium* strains LBA 4404 and EHA 105 harbouring the binary plasmid PGA 472 as well as co-integrate vector (PGV 2260-PGSFR 280)**

	Control 1			Control 2			Infected leaf segments								
							LBA 4404			EHA 105			Co-integrate vector		
Explant Source	No. of explants	No. of explants callusing	Frequency of callusing (%)	No. of explants	No. of explants callusing	Frequency of callusing (%)	No. of explants	No. of explants callusing	Frequency of callusing (%)	No. of explants	No. of explants callusing	Frequency of callusing (%)	No. of explants	No. of explants callusing	Frequency of callusing (%)
Cotyledon	10	9	90	10	0	0	14	3	21.4	16	3	19.0	14	0	0
Primary leaves	20	19	95	10	0	0	14	0	0	16	0	0	18	0	0

originally white in colour turned progressively green. Uninfected tissues kept on selection medium failed to callus and died with in 3-4 weeks. The callusing was delayed by 5-6 weeks in case of the infected tissues and the callus was very friable and white in colour, as compared to the control.

Although virulence system of different *Agrobacterium* strains share common functions they are not identical and some may give rise to more transformation on certain plant species, than other (Byrne *et. al.*, 1985; Filliate *et. al.*, 1987). This efficiency is related to the efficiency of particular *vir* gene in the TDN transfer process than to do with the oncogenity of the TDNA involved. However, in the present study even though the strain EHA 105 had a more virulent *vir* G gene it did not reflect in the efficiency of transformation. Similarly the co-integrate vector which is reported to be very effective in tomato system also did not result in transformation of black pepper. In the present study only the cotyledon explants were found amenable to transformation.

One advantage of the binary vector system is that they can be mobilized into any *Agrobacterium* host, not just hosts containing Ti plasmid with homologous DNA. The co-integration system suffers from the fact that a specific disarmed Ti plasmid must be used to provide helper function and thus host range of the plant species that may be transformed is limited by the properties of the plasmid in which the deletion were made (Rogers *et al.* 1987). This might be the reason why co-integrate vectors are efficient only in certain plant species.

Confirmation of the transformation was done by phenotypic assay of the transformed calli (Table II.) Control and the transformed calli were grown on selection medium containing various concentrations of kanamycin viz. 50, 100, 150, 200 and 300 $\mu g\ ml^{-1}$ along with 500 $\mu g\ ml^{-1}$ carbenicillin. Sasikumar and Veluthambi (1994) while assaying the kanamycin sensitivity of cultured black pepper tissues observed that callus formation was completely inhibited at 50 $\mu g\ ml^{-1}$ above concentration of kanamycin in the medium suggesting that 50 $\mu g\ ml^{-1}$ kanamycin is the minimum concentrations needed to select the transformed tissues of black pepper. Transformed calli remained fresh and proliferated upto 150 $\mu g\ ml^{-1}$ kanamycin in the present study. (Fig. 2) Above this concentration, callus death was noticed after 25 days of culture. However, in case of the control calli no callus proliferation was observed above 50 $\mu g\ ml^{-1}$ kanamycin. Thus, the higher kanamycin tolerance of the transformed callus testifies the transformation of the tissues by the binary plasmid pGA 472 containing the NPT-II gene conferring resistance to kanamycin. The ability of the transformed tissue to grow under dominant selection marker such as kanamycin or hygromycin is considered as one of the confirmations for transformation (Walden, 1985).

Table II. Phenotypic assay for confirming transformation in black pepper

Kanamycin concentration ($\mu g ml^{-1}$)	Control Callus	Transformed Callus
50	+	+
100	–	+
150	–	+
200	–	–
250	–	–
300	–	–

+ indicate growth and proliferation of calli

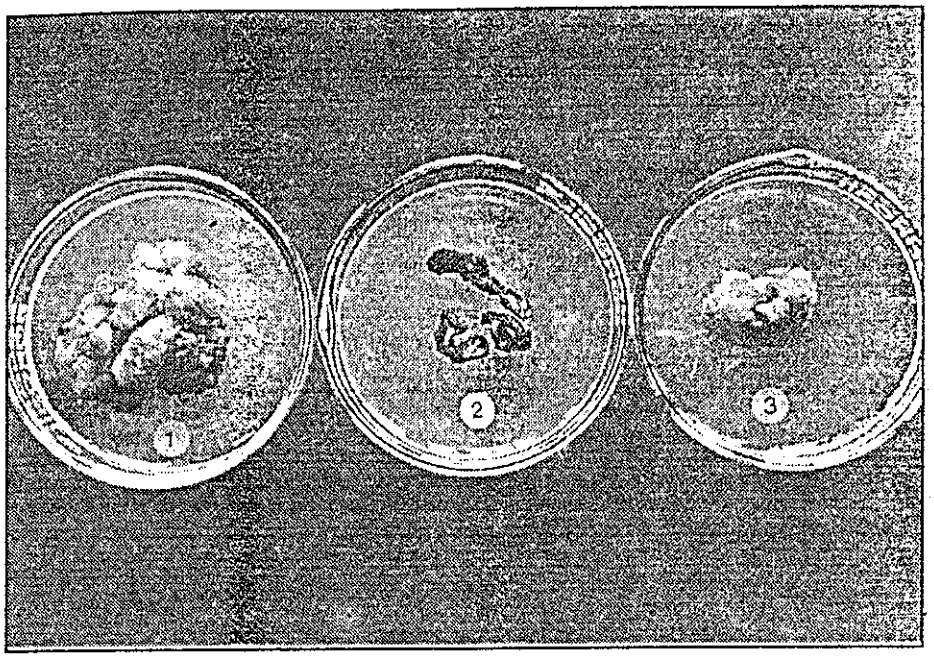

Fig.1 Transformation in black pepper. Cotyledon explants of black pepper cv. Karimunda were transformed using *Agrobacterium tumefaciens* strains harbouring pGA 472 untransformed cotyledon segments callusing in kanamycin free medium (1), untransformed cotyledon segments left on selection medium with inhibitory levels of kanamycin (2), and cotyledon segments transformed with *Agrobacterium* left on selection medium (3).

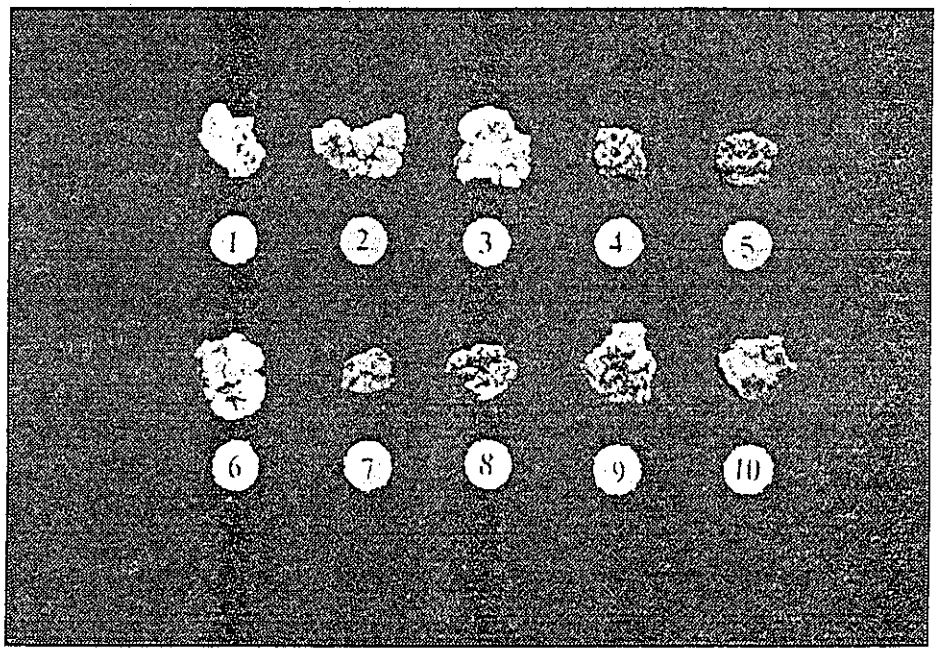

Fig. 2. Phenotypic assay for confirmation of transformation. Transformed calli growing in 50, 100 and 150 mg m l^{-1} kanamycin (1, 2 and 3 respectively). Transformed calli did not grow in 200 and 300 mg ml^{-1} kanamycin (4) & (5). Control calli grew only at 50 mg ml^{-1} of kanamycin (6). Control calli death was evident at kanamycin concentrations of 100 mg ml^{-1} and above (7), (8), (9) and (10) - indicate callus death

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