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Transformation of black pepper (*Piper nigrum* L) using *Agrobacterium* Ti plasmid based vectors

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ABSTRACT

Cotyledon expiants of the black pepper cultivar 'Karimunda' infected with the Agrobacterium tumefaciens strain LBA 4404 harbouring the binary vector PGA 472 and cultured on MS selection medium containing 50μg ml^{-1} kanamycin and 500μg ml^{-1} carbenicillin resulted in 20% explants callusing. Phenotypic assay for confirmation of transformation using higher concentrations of the selective marker, kanamycin, revealed the high degree of tolerance of the transformed tissue as compared to the control.

INTRODUCTION

Black pepper (*Piper nigrum* L.) is an important spice crop of many tropical countries. Black pepper of commerce is the dried berries of this perennial vine. Sufficient genetic variability for many of the agronomic and quality traits is encountered in this crop and exploitation of such variability has led to the development of many superior varieties. However, genetic variability in cultivated varieties for one of the most important and serious problems being faced by the producers i.e., foot rot disease (quick wilt) caused by *Phytophthora capsici*, is rather nil at present. Hence coventional breeding techniques elude solution to this problem at present.

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The advent of r-DNA techniques will allow the introduction of foreign gene or genes from related species, conferring resistance/tolerance against this disease, to established cultivars.

Agrobacterium mediated transformation of plants remains the simplest and most reliable means for introducing foreign DNA into the genome of dicotyledonous plants (Corbin and Klee, 1991). In order for genetic modification using *Agrobacterium* to be successful, an effective transformation and regeneration system must be in place. In this regard we report the result of first ever transformation study done in black pepper using *Agrobacterium* strain LBA 4404 harbouring the binary vector PGA 472 with neomycin phosphotransferase (NPT II) as the selectable marker.

MATERIALS AND METHODS

(i) **Plant material:** Cotyledons and primary

leaves of 3-4 month old black pepper cultivar 'Karimunda' raised in sand filled basin as the explant.

(ii) **Surface sterilization:** Cotyledons and primary leaves were separated, washed thoroughly in running tap water and sterilized sequentially in:

A. 70% ethanol for 5 minutes followed by thorough washing in sterilized water.
B. 40% v/v sodium hypochlorite plus few drops of tween 80 for 5 minutes followed by thorough washing in sterilized water.
C. 0.1% $HgCl_2$ for 3 minutes.

Finally the explants were washed thoroughly in sterile water, transferred to a sterile petriplate with filter paper and cut horizontally into segments of size 0.2 x 1.5 cm after cutting off the edges.

(iii) **Culture conditions:** The explants were cultured on a medium containing Murshige and Skoog salts and vitamins, 30g l^{-1} sucrose plus BAP 3 mg l^{-1}, NAA 2mg l^{-1} and 2,4-D 1mg l^{-1}. The medium was adjusted to pH 5.7 autoclaved and dispensed as 25ml aliquots to 100ml conical flasks. There were 4 flasks/treatment with 3-4 explants each to a single flask. All cultures were maintained at 25±1°C under 16h photoperiod.

(iv) **Transformation:** The disarmed *Agrobacterium tumefaciens* strains LBA 4404 containing the binary plasmid PGA 472 was used for transformation. PGA 472 contains a neomycin phosphotransferase II (NPT II) gene under the control of nopaline synthase promoter. Bacteria were grown for about 35hr. (reaching 0.7 to 1.0.D) in AB minimal medium containing 2.5 µg ml^{-1} tetracycline at 30°C with shaking. Preincubated explants (for 2 days) in drug free medium were submerged in the bacterial culture, with gentle shaking of the plate, for 2-3 minutes, blotted dry on a sterile filter paper and co-cultivated on fresh medium (without drug) for another 2 days. Explants were then transferred to selection medium containing 500 μg^{-1} kanamycin (Kanamycin monosulphate) and 500 µg mg^{-1} carbenicillin and subcultured onto fresh selection medium periodically. Control (Control 1) explants did not receive the infection and they were left in the same medium but without the antibiotics. Another set of uninfected control (Control-2) was left on selection medium with the inhibitory levels of the drug.

(v) 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. Kanamycin concentrations of 50, 100, 150, 200 and 300 µg ml^{-1} with 500µg ml^{-1} carbenicillin were used in this study. Two sets of flasks were kept in each case. Culture medium and culture conditions remained the same described above. Growth and proliferation of calli were monitered.

RESULTS AND DISCUSSION

About 20% callusing was observed in cotyledon explants of black pepper 'Karimunda' infected with the *Agrobacterium* strain LBA 4404 harbouring the binary vector PGA 472 and cultured on selection medium containing 50 µg ml^{-1} kanamycin and 500µg ml^{-1} carbenicillin (Table 1 and Fig. 1). The callusing was delayed by 5-6 weeks in case of the infected tissues and it was very friable and white coloured. Even though infected explants from primary leaves remained alive even after 3 months of culturing in selection medium, no appreciable callus development could be observed in this material. However, slight swelling and bulging of the tissues were observed. Control explants on non selective medium (Control-1) in both the cases had about 90% callusing. The control calli which was originally white in colour turned progressively green. Uninfected tissues kept on selection medium with inhibitory levels of kanamycin failed to callus and died within 3 weeks.

Confirmation of the transformation was done by Phenotypic assay of the transformed calli (Table-2). Control and transformed calli were grown on selection medium containing various concentrations of kanamycin viz. 50, 100, 150, 200 and 300µg ml^{-1} alongwith 500µg ml^{-1} carbenicillin. Transformed calli remained fresh and proliferated up to 150 µg ml^{-1} kanamycin (Fig.2). Above this concentration callus death was noticed after 25 days of culture. However in case of the control calli no callus proliferatoin was oberved above 50µg ml^{-1} kanamycin. Thus the higher kanamycin tolerance of the transformed callus testifies the transformation of the tissue by the binary plasmid PGA 472 containing the NPT II gene conferring resistance to kanamycin. The ability of the transformed tissues to grow under dominant selection marker such as kanamycin or hygromycin is considered as one of the confirmations for transformation (Walden, 1988).

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Table 1: Frequency of callusing from black pepper explants infected with the *Agrobacterium* strain LBA 4404 harbouring the binary plasmid PGA 472 and controls.

	Control 1			Control 2			Control 3		
Explant source	No. of explants	No. of explants callusing	Freq. of callusing (%)	No. of explants	No. of explants callusing	Freq. of callusing (%)	No. of explants	No.of explants callusing	Freq. of callusing (%)
Cotyledon	10	9	90	10	0	0	14	3	21.4
Primary leaves	20	19	95	10	0	0	14	0	0

Table 2: Phenotypic assay for confirming transformation of black pepper

Kanamycin concentration (µg ml^{-1})	Control callus	transformed callus
50	+	+
100	-	+
150	-	+
200	-	-
300	-	-

+ indicates growth and healthy calli - indicates callus death

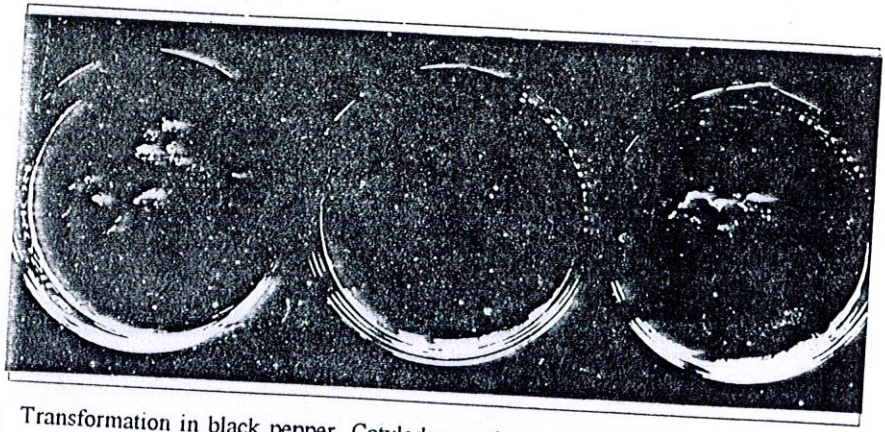

Fig 1: Transformation in black pepper. Cotyledon explants of black pepper Karimunda 'cv' were transformed using *Agrobacterium tumefaciens* harbouring PGA 472. Untransformed cotyledon segments callusing in kanamycin free medium 1, untransformed cotyledon segments left on selection medium with inhibitory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

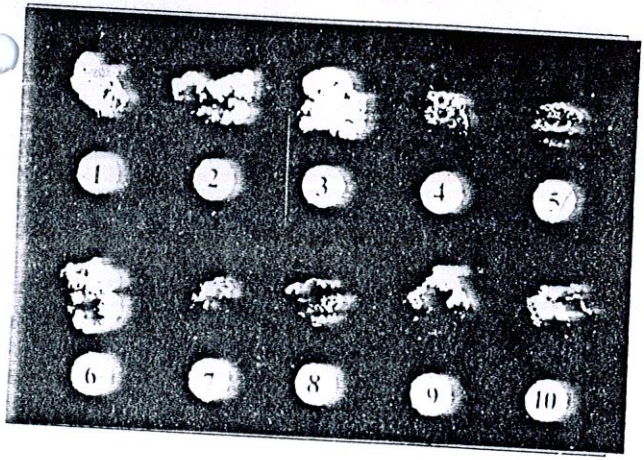

Fig. 2. Phenotypic assay for confirmation of transformation. Transformed calli growing in 50, 100 and 150 µg ml^{-1} kanamycin (1, 2 and 3 respectively). Transformed calli did not grow in 200 and 300 µg ml^{-1} of kanamycin (4 & 5). Control calli grew only at 50 µg ml^{-1} of Kanamycin (6). Control calli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