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Putative transgenic plants through *in planta* transformation against *Phytophthora* foot rot in black pepper (*Piper nigrum* L.)

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Black pepper (*Piper nigrum* L.), popular as 'king of spices' is one of the important export earning spice crop of India. One of the serious reasons causing the low productivity of black pepper during the last decades was the incidence of foot rot disease caused by the *Phytophthora capsici* in the pepper plantations of Kerala, the major pepper producer state in India. Since all the available cultivated varieties are susceptible to this disease, an attempt was done for utilising the disease resistance available in the related pepper species by adopting a different plant transformation approach. The *in planta* transformation via pollen tube pathway was done in the black pepper variety Panniyur-2, using the total exogenous DNA of *Piper colubrinum* , a wild relative species of Piper resistant to the dreaded foot rot disease caused by *Phytophthora capsici*. The resulting putative transformant seeds were germinated *in vitro* by embryo rescue technique. These germinated putative transformants were later subjected to *in vitro* multiplication, elongation and rooting. These cultures were screened *in vitro* in the rooting phase by incorporating the toxic culture filtrate of the pathogen *P. capsici* in the rooting media. The rooted putative transformant plantlets were hardened and screened artificially for disease tolerance under ex vitro conditions. The survived seedlings were planted out. The RAPD analysis of the plantlets with the decamer primers OPA 08 and OPG 08 have shown variation in banding pattern compared to the DNA recipient parent *P. nigrum* variety Panniyur-2.

Key words : Black pepper, *Piper nigrum*, *Piper colubrinum*, *In planta* transformation, Pollen tube pathway transformation, *In vitro* germination, Embryo rescue, Disease screening, RAPD analysis.

INTRODUCTION

The dried mature seeds of black pepper, known as the king of spices or black gold is the most widely used spices in the world. The projected world consumption of black pepper is estimated to be around 2.3 lakhs and 2.80 lakh metric tons by 2010 and 2020, respectively. One of the major reasons for the perceptible decline during the last few years of pepper production is the foot rot disease of black pepper caused by the pathogen *Phytophthora capsici*. To bridge the gap between demand and consumption a modest hike of 1.0 lakh metric tons is required for the next two decades by large scale cultivation of tolerant or resistant cultivars against the biotic stresses especially *Phytophthora* foot rot diseases (Ravindran, 2000). All *Piper nigrum* cultivars are susceptible to this serious disease and have to be chemically protected. The demand for clean spices free from chemical residues and attempts to reduce the cost of cultivation led to intensive research for host-plant resistance. *Piper colubrinum*, known as Brazilian thippali and a distantly related species from South America, is resistant to *Phytophthora* (Ravindran, 2000). However, natural genetic barriers have limited the resources available from other species.

Genetic transformation is a useful approach to overcome the problem of sexual barriers and to introduce desirable genes from other sources into plants for cultivar development. The unique *in planta* transformation method to produce high frequency genetic transformation by applying exogenous DNA to pollen grains and to stigma at the time of pollination is more adequate in this aspect. This approach may be imitating the conventional hybridization with a touch of advance biotechnology. The repeated *in planta* transformation using F_1 putative transformant like back cross method may be helpful to evolve *Phytophthora* foot rot resistant variety of black pepper. Increased selection pressure through *in vitro* technique may also be advantageous in confirming the tangible result in the perennial spice crop like black pepper. In the present study, an *in planta* transformation was attempted in *Piper nigrum* var Panniyur-2 by adopting pollen tube pathway transformation using total exogenous DNA of *P. colubrinum*. Panniyur-2 is the open pollinated variety selected from Balankotta.

MATERIALS AND METHODS

Six bush pepper plants of variety Panniyur-2 (3 years old

and started profuse flowering) and *Piper colubrinum* plants were selected for the study. Panniyur-2 (Fig. 1a) was the DNA recipient plant and *Piper colubrinum* plants (Fig.1b) were used as DNA source plant. DNA was isolated from *Piper colubrinum* by the CTAB protocol of Rogers and Bendich (1994).

Delivery of DNA:

The crossing technique making use of the protogyny reported by Ravindran *et al.* (1981) was used for this experiment. The procedure of Zhou *et al.* (1988) and Lei *et al.* (1995) was used with some modifications for the pollen tube pathway transformation. For this, freshly opened anthers were collected into a small vial and few drops of distilled water was added to the vial shaken well and 1.0 ml of the pollen suspension was added to the DNA solutions so that the final DNA concentration is 50 µg/ml. Then it was incubated for 5-10 minutes, the pollen DNA suspension was applied using a syringe and was brushed on to the stigma with a clean camel hair brush (Fig. 1c). The process of pollination was continued for a few days till anther emergence. When the artificial pollination stopped, the unfertilized portion of the spike was trimmed off and the remaining portion of pollinated spike was covered with paper bag.

In vitro seed germination through Embryo rescue:

The berries (3/4th mature to mature) were excised from the artificially fertilized spikes. The seeds were washed thoroughly with water containing few drops of teepol and 0.1 per cent Bavistin. These pre-treated seeds were surface sterilized with $HgCl_2$ (0.1%) for 10 minutes under aseptic condition of laminar hood. Then the seeds were washed to free of $HgCl_2$ by rinsing with distilled water for 3 to 4 times and drained on sterile blotting paper. The fertilized ovules or embryos with bits of endosperm were dissected out aseptically using sterile scalpel and blade and were inoculated on the sterile medium. The media used for embryo rescue and regeneration (Nazeem *et al.*, 1990, 1992 and 2004; Lissamma, 2007) are given in Table 1.

Screening for disease resistance:

In vitro screening:

Pure culture of *Phytophthora capsici* isolated from the infected leaves of black pepper was inoculated to liquid Ribiero medium and incubated for 10 to 14 days. The toxic culture filtrate was collected by filtering through Whatmann No. 1 filter paper. The concentrated culture filtrate was added to the rooting medium @ 7.5 % v/v and the elongated shoots were inoculated. Then the cultures were incubated at 26±2°C for 16 h photoperiod with 1000 – 1500 lux supplied by fluorescence tubes. The number of roots produced, length of roots and thickness of the roots were observed 40 days after inoculation and compared with medium control.

Table 1 : Media used for embryo rescue and *in vitro* regeneration of putative transformants

Media used	Media composition	Stage of culture
M_1	½ MS + 30gl^{-1} sucrose + 0.1 gl^{-1} inositol + 0.75 gl^{-1} agar	Embryo rescue
M_2	½ MS + BA (1 mgl^{-1}) + IAA (1 mgl^{-1}) + 30gl^{-1} sucrose + 0.1 gl^{-1} inositol + 0.75 gl^{-1} agar	Embryo rescue and multiple
M_3	½ MS + BA (0.2 mgl^{-1}) + IAA (0.1 mgl^{-1}) + 30gl^{-1} sucrose + 0.1 gl^{-1} inositol + 0.75 gl^{-1} agar	Elongation
M_4	½ MS + IBA (5 mgl^{-1}) + 10 gl^{-1} sucrose + 0.75 gl^{-1} agar	Rooting

Ex vitro screening for disease resistance:

The 5 mm diameter mycelial discs of 7 to 10 day old cultures of *Phytophthora capsici* on carrot agar media were used for inoculating leaves of *P. nigrum* var Panniyur-2, *P. colubrinum* and the *in vitro* screened putative transformants. Mycelial discs were placed on the under surface of leaves after giving mild pinpricks with sterile needle and over which moist cotton was placed. High humidity was maintained throughout 48 h period by covering with polythene bags and the inoculated plants were watered regularly. Disease scoring was done by the modified scoring technique of Kheu and Khew (1980) reported by Shylaja (1996) (Table 2). The leaf diameter after 48h infection was measured and the individual plants are scored. Then the lesion diameter score of each class was calculated by multiplying individual score of each class with the percentage of plants on each class.

Random Amplified Polymorphic DNA (RAPD) analysis:

RAPD analysis of putative transformants and parents

Table 2 : Leaf lesion diameter scores

Class	Leaf lesion diameter in cm	Score
1.	< 0.5	1
2.	0.5-1.0	2
3.	1.1-1.5	3
4.	1.6-2.0	4
5.	>2.0	5

was carried out using the 2 selected random primers OPA 08 (GTGACGTAGG) and (TCACGTCCAC). DNA was extracted from young leaves, using the mini-prep cetyl trimethyl ammonium bromide (CTAB) method (Khan *et al.*, 2004). Polymerase Chain Reaction (PCR) was carried out in a 25µl reaction mix containing 1.5 µl of 10 picomol of decamer random primers (Operon technologies, Inc., Alameda, CA, USA), 1µl dNTP mix (10 mM each), 0.3 µl Taq DNA polymerase (3 unit/µl) and 25ng of genomic DNA. PCR amplification conditions were initial denaturation of 94°C for 4 min followed by 39 cycles of 94°C for 1min (denaturation), 35°C for 1min (annealing), 72°C for 2 min (extension), and a final extension at 72°C for 5 min. The RAPD fragments were separated by electrophoresis on 1.2 per cent agarose gel and visualized with ethidium bromide.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below:

Pollen tube pathway transformation:

Pollen tube pathway transformation is a method that delivers foreign gene directly into germ-line cells, combining genetic engineering and convention breeding to achieve crop improvement. Introduction of exogenous DNA into a plant embryo through the pollen tube pathway after pollination was first reported by Zhou *et al*. (1983) in cotton. The male gametophyte or pollen grain has major advantages over the use of plasmid as vector. The most important advantage is efficiency. Germination and incubation of pollen can be readily achieved in the field and pollination followed by selection is the standard breeding tools for plant improvement. The germinating pollen grains incubated with alien DNA affect fertilization, and induce directional mutation in the genome of the zygote which is expressed in the resulting offspring and their descendents. There are two technological handicaps encountered by the commonly applied transformation methods which are overcome by using the pollen tube pathway procedures *i.e*. Large pieces of DNA segments, even the genomic total DNA can be used and introduced with the pollen tube pathway transformation overcoming the size limitation problems of exogenous DNA. The capacity of the pollen tube pathway procedure in transferring multigenic qualitative traits from one organism to another was vividly illustrated in Soybean 'Heisheng 101' from which the average yield increased nearly 50 per cent (Hu and Wang, 1999). Good quality DNA of *P. colubrinum* (Fig. 2a and 2b), without any RNA contamination and having absorbance ratio 260/280 ranging from 1.8 to 2.0 was used for pollen treatment. The details of pollination and the seed set obtained after the pollen treatment are given below

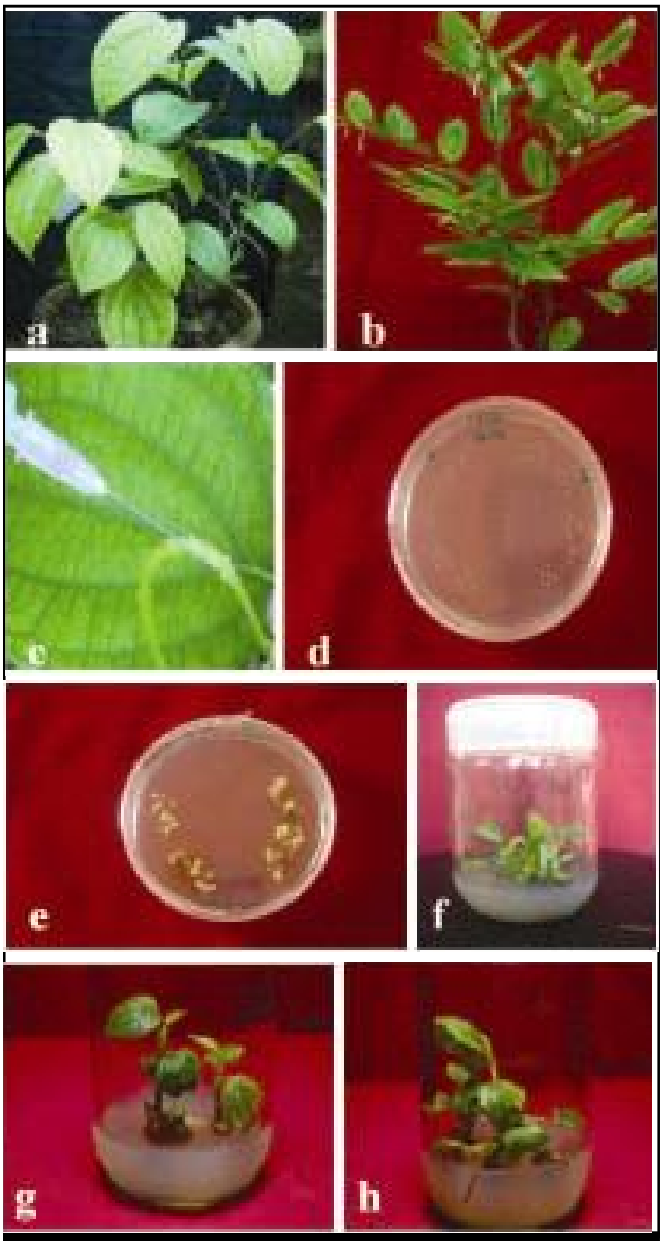


Fig. 1 : Embryo rescue of F_1, putative transformants, (a) *Piper nigrum* var P_2, (b) DNA donour-*P. colubrium*, (c) Pollination, (d) Embryo rescue, (e) Embraya germination, (f) Multiple shoot induction, (g) Elongation of shoots, (h) Rooting

Embryo rescue:

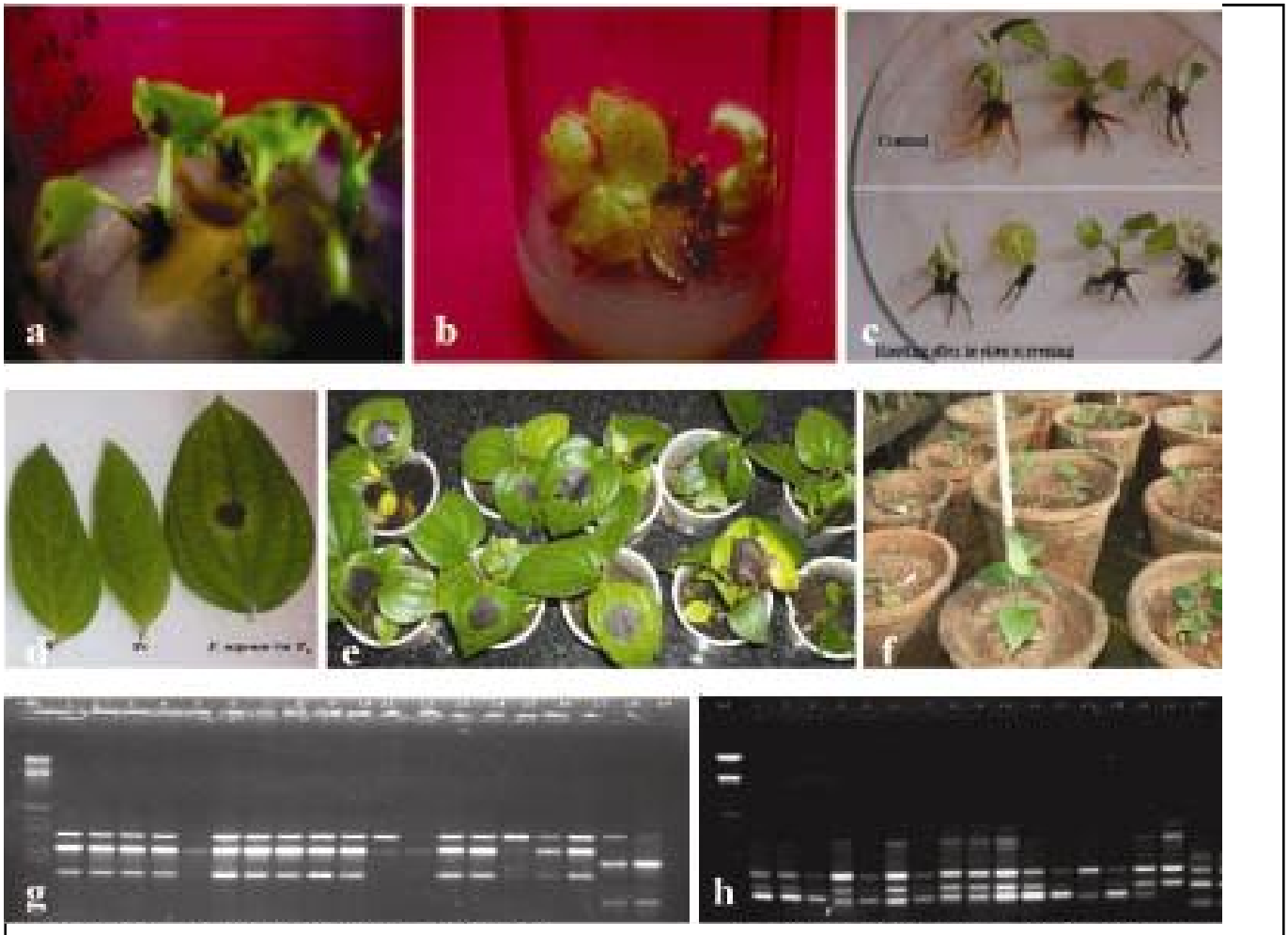
The embryo rescue of suspected intragenic F_1 seeds was inoculated to M_1 medium. It is comparatively easier to excise embryos with endosperm (Fig. 1d and 1e) than

Table 3 : Pollination and seed set in DNA treated spikes of *P. nigrum* var Panniyur-2

Total number of pollinations per spike	Total number of spikes treated	Total number of spikes matured	Seed set (%)	Total number of seeds obtained
3	75	52	69.33	1300

the embryo alone from the three-fourth mature seeds. The germination of embryo in the M_2 medium was 71.19 per cent compared to 36.36 per cent in M_1 medium (Table 4). Immature embryos generally fail to grow on basal medium and exhibited low germination and subsequent growth of intragenic plants. Bhojwani and Razdan (2006) and Raghavan (2004) already reported the more elaborate nutritional requirements of the immature embryos than those of the mature embryos. The germinated seedlings from M_1 medium were healthy and have shown further regeneration compared to the delayed or absence of regeneration from M_1 medium. The study conducted at KAU (Cheriyan, 2000 and Lissamma, 2007) reported 37 per cent germination of embryo from the ripe berries of black pepper *in vitro*.

Then the germinated putative transformants were initiated 4 to 5 multiple shoots at culture duration of 30 to 45 days (Fig.1f). The individual shoots were separated and transferred to the elongation medium (M_2) (Fig.1g) followed by the rooting medium (M_3) (Fig.1h). The response of cultures during *in vitro* germination and subsequent phases of growth in different media are summarized in the Table 5.



M-Marker DNA, 1-3 *Piper nigrum var. Panniyur-2*, 4-17 Putative transformants, 18-19 *Piper colubrium*

M-Marker DNA, Lane 1, 2-*P. nigrum* var P_2 Lane 18-19 *P. Colubrinum*, Lane 3-17 puntative Transformatns, 19-Blank

Fig. 2 : *In vitro* screening, ex vitro screening and molecular diagnosis (a) Leaf necrosis, (b) Drying of cultures, (c) Reduction in root thinks, (d) Symptoms produced by *Phytophithora capsici* on *P. colubrium* and *P. nignum var* P_2, (e) Leaf lesions on putative transformants, (f) Survived resistant/intrugenic plants (g) & (h) RAPD profiles using OPA-08 & PG-08 primers

Table 4 : *In vitro* germination of excised embryos from the three-fourth mature seeds

Media used	Total number of seeds taken	Number of embryos excised	Germination percentages	Time taken for germination	Final survival percentage	Nature of response
M_1	600	200	36.36	14-20	60	Germination of weak seedlings and delayed regeneration
M_2	600	196	71.19	14-25	72.5	Germination and further regeneration

Table 5 : Response of cultures during *in vitro* germination

Media	Response culture	Percentage recovery	Culture duration	Subculture cycles followed
M_1	Embryo germination	66	10-25	3
M_2	Embryo germination and multiple shoot induction	60	25-30	2
M_3	Elongation	80.32	30-45	2
M_4	Rooting	88.5	14-25	2

Disease screening:

In vitro screening:

The F_1 putative transformants inoculated to the toxin added media could able to survive and produced roots in 69.33 per cent of cultures. The symptoms observed during the *in vitro* screening were leaf necrosis, drying of the cultures and reduction in thickness of roots (Fig. 2a,2b and 2c). Jin- Peng *et al.*, 2002 reported the necrosis and death of cultures in *Piper nigrum* under *in vitro* screening with toxic culture filtrate of *P. capsici*. The response of cultures during *in vitro* screening is shown in Table 6. The plantlets survived after *in vitro* screening were hardened and planted out.

Ex vitro screening:

The leaf lesion diameter scores after disease screening are shown in Table 7 and 8. Among the ex vitro screened putative transformant plantlets, the highest percentage of seedlings (42.15) was in class 2, followed by 29.41 per cent in class1. The total score was 206.65 for putative transformants. Among the parent plant *P. nigrum* var Panniyur-2, the 60 per cent of plants were in class 2 followed by class 3(22 %). But in putative transformants, the percentage of plants in class 2 was reduced and in class1 (less than 0.5cm diameter), the percentage of plants increased. This shows an increase in tolerance level of the putative transformants. The parent

Table 6 : Response of putative transformants during *in vitro* screening with toxic culture filtrate of *Phytophthora capsici*

Category of cultivars	Number of cultures used	Percentage recovery of cultures	Percentages of cultures with leaf necrosis	Average number of roots	Average of length of roots (cm)	Nature of response
Putative transformat	75	69.33	22.30	3	1.22	Thick, and healthy roots
P. nigrum var P_2 (Open pollinated)	40	65	25.33	3	1.24	Lean and lanky roots

Table 7 : Disease indexing / scoring after artificial inoculation in putative transformants

Plant category	Total number of plantlets tested	Percentage of plants in each class				
		Class 1 (<0.5 cm)	Class 2 (0.6-1.0 cm)	Class 3 (1.1-1.5 cm)	Class 4 (1.6-2.0 cm)	Class 5 (>2.0 cm)
Piper nigrum var. P_2	50	10.00	60.00	22.00	8.00	0
P. colubrinum	10	0	0	0	0.00	0
Putative transformants	102	29.41	42.15	20.58	7.8	0

Table 8 : Leaf diameter scores after disease screening

Plant category	Total score	Leaf diameter score in each class				
		Class 1 (<0.5 cm)	Class 2 (0.6-1.0 cm)	Class 3 (1.1-1.5 cm)	Class 4 (1.6-2.0 cm)	Class 5 (>2.0 cm)
Piper nigrum var. P_2	228.00	10.00	120.00	66.00	32.00	0
P. colubrinum	0	0	0	0	0	0
Putative transformants	206.65	29.41	84.30	61.74	31.20	0

plant *P. nigrum* var Panniyur-2 recorded a highest leaf lesion diameter score (228). The *P. colubrinum* had not shown any symptoms (Fig. 2d and 2e.)

The disease screened plantlets showed leaf drop, node infection and collar rot followed by the artificial inoculation of pathogen. But the final survival (Fig. 2f) of plants was 39.21 per cent in putative transformants.

RAPD analysis:

Most of the initial reports of pollen tube pathway transformation used total genomic DNA for transformation. In these studies, the treated plants were monitored and compared with untreated controls for variations in plant morphology, fertility, pest resistance, seed composition and peroxidase isozyme expression patterns (Lei *et al.*, 1994, 1995; Liu *et al.*, 1992, 1997; Zhao *et al.*, 1995). The standard transformation confirmation procedures the Southern analysis cannot be applied when exogenous chromosomal or genomic total DNAs are used in transformation. An alternative procedure, the RAPD was used in black pepper associated with exogenous genomic total DNA transformation. Variation in RAPD analysis of treated soybean plants correlated with the introduction and integration of the donour DNA from wild soybean via the pollen tube pathway transformation (Lei *et al.*, 1994).

Hybrid identification and confirmation of the ex vitro and *in vitro* intragenic or putative transformants were tested using the primers OPA08 (Pradeepkumar *et al.*, 2005) and OPG08 (Vanaja *et al.*, 2007) with respect to parent *P. nigrum* and *P. colubrinum* (Fig.2g). The 33.89 per cent of the putative transformants were exhibited variation from the parent plant $P_{2.}$ For the primer OPA 08, the extent of variability of the seedlings from the parent *P. nigrum* var P_2 were 9 per cent for the putative transformants (Fig.2h). The similarity of banding pattern of most of the *in vitro* and ex vitro putative transformants may substantiate the successful efforts for developing intrageneic transformants against *Phytophthora* foot rot resistance in black pepper. But the putative transformants have not shown any *P. colubrinum* specific bands for the above primers.

The putative transformant plantlets showed variation in the level of resistance to *P. capsici* both *in vitro* and ex vitro screening. The variation in banding pattern observed in the putative transformants leads to a conclusion that it is possible to occur through *in planta* method via pollen tube pathway techniques. The level of resistance can be further improved by the repetitive *in planta* transformation as in back cross method of the conventional hybridization. But *P. colubrinum* specific bands were absent in these putative tranformant plantlets. Therefore, more number of primers can be used for screening the putative transformants for the confirmation of transformation and disease resistance. Whether these plants can show the same resistance in the field needs to be further investigated. The 1,3 b-glucanase activity and southern blot hybridization can be employed for the reliable confirmation of disease resistance.

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Abbreviations:

mg- milligram, *P.nigrum- Piper nigrum*, *P. colubrinum -Piper colubrinum*, DNA- Deoxyribo Nucleic Acid , ml -Microlitre, h- hour, MS medium- Murashige and Skoog medium, BA - Benzyl Adenine , IAA - Indole Acetic Acid , IBA-Indole Butyric AcidPCR- Polymerase Chain Reaction, ng- nanogram, $HgCl_{2-}$ Mercuric chloride, KAU- Kerala Agricultural University, RAPD –Random Amplified Polymorphic DNA

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