Somatic embryogenesis and transgenic development in black pepper for delayed infection and decreased spread of foot rot caused by *Phytophthora capsici*

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(Manuscript Received:16-07-13, Revised:30-09-13, Accepted: 14-10-13)

Abstract

Phytophthora capsici causing *Phytophthora* foot rot is the most important pathogen of black pepper (*Piper nigrum* L.). With a view to develop transgenic plants with resistance to this pathogen, *Agrobacterium* mediated genetic transformation using a PR 5 (pathogenesis related) gene '*osmotin*' was attempted. Initially protocols for plant regeneration and somatic embryogenesis were optimised. Murashige and Skoog (MS) and woody plant media (WPM) supplemented with benzyl adenine (BA) (0.05-1 mg l^{-1}) and thidiazuron (TDZ) (0.05-1 mg l^{-1}) was found to be ideal. Plants could be successfully regenerated from tender leaf, shoot tip and hypocotyl explants of both mature and juvenile tissues. Somatic embryogenesis was also induced successfully from tender leaf explants of *in vitro* established mature tissues using the same medium. Successful transformation of leaf explants from these somatic embryo derived plants using *Agrobacterium* strain pGV 2260 carrying '*osmotin*' under the control of CaMV 35S promoter was achieved. About 50 putative transgenic plants were obtained and planted in the green house. The gene transfer was confirmed by polymerase chain reaction (PCR) using *osmotin* and *npt II* specific primers. Infection with *P. capsici* on detatched leaves of putative transgenics showed delayed infection and decreased rate of disease spread indicating differential expression of *osmotin*. Stem and leaf inoculated plants showed survival, while root inoculated plants succumbed to infection and died. It can be inferred that *osmotin* confers resistance to infection by *Phytophthora capsici* in black pepper.

Keywords: *Agrobacterium,* black pepper, osmotin, *Phytophthora* foot rot, somatic embryogenesis

Introduction

Black pepper (*Piper nigrum* L.) is considered as 'king' of spices and is an important part of coastal ecosystem of western coast of India. The production of black pepper is not increasing irrespective of the development of many high yielding varieties. This is mainly due to the prevalence of the epidemic disease, *Phytophthora* foot rot caused by *Phytophthora capsici*, a fungus like oomycete pathogen. In many areas, cultivation of black pepper has become difficult due to the prevalence of this disease. Plant protection methods using chemicals have resulted in partial success only. Besides, there is an overwhelming demand for clean spices free from chemical residues. All the available cultivated varieties are susceptible to this disease and two decades of continued efforts have led to identification of only a few *P. capsici* tolerant lines (Bhai *et al*., 2010). The only source of resistance is from a very distant species and a different cytotype, *Piper colubrinum*. Though there is report about interspecific hybrids of *P. nigrum* and *P. colubrinum* (Vanaja *et al*., 2008), it is not repeatable. Piper being a perennial crop, the conventional breeding programmes are also delayed significantly. Thus developing novel alternate strategies is required to

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produce disease resistant varieties and thereby producing a chemical residue free pepper in the market.

The most significant development in the area of varietal development for disease resistance in plants is the use of techniques of gene isolation and genetic transformation (Grover and Gowthaman, 2003). The most important event occurring during fungal pathogenesis in plants is induction of genes encoding antifungal PR proteins. Transgenics overexpressing PR 5/osmotin have been implicated in fungal resistance (El-Kereamy *et al*., 2011) and delayed development of disease symptoms (Li *et al*., 1999; Liu *et al*., 1994) in many plants. Induction of PR proteins in *P. capsici* infected tissues of black pepper was confirmed by SDS-PAGE (Stephen *et al*., 2001). Putative transgenic black pepper plants developed by *in planta* transformation showed differential response to infection by *P. capsici* (Asha and Rajendran, 2010). Transgenic plants constitutively expressing more than one PR protein gene showed better resistance levels than those with a single gene (Jongedijk *et al*., 1995). Two osmotin isoforms cloned from *P. colubrinum* and expressed in *E. coli* showed significant antifungal activity of the recombinant protein on *P. capsici* (Mani *et al*., 2012). Hence we have attempted to develop transgenic black pepper carrying *osmotin* through *Agrobacterium* mediated genetic transformation for developing plants resistant to *P. capsici*.

Somatic embryogenesis is a desirable method in genetic transformation since they occur from somatic tissues through asexual process. However, like zygotic embryos, somatic embryos can be germinated to form plants. Another important feature of the somatic embryogenesis in the context of transgenic development is that they can multiply themselves to produce many more somatic embryos through a process referred to as secondary or repetitive embryogenesis. There are several protocols for somatic embryogenesis from mature tissues in black pepper. Biju *et al*. (1996) reports somatic embryogenesis from zygotic embryo derived callus in black pepper. Plant regeneration through direct somatic embryogenesis from micropylar tissues of germinating seeds and scaling up through high-frequency cyclic secondary somatic embryogenesis have been described (Nair and Gupta, 2003). However, reports on somatic embryogenesis in black pepper from juvenile tissues are lacking, which will be ideal in terms of genetic fidelity and stability as well as rapid multiplication.

Genetic transformation in black pepper showed retarded regeneration potential from mature tissues due to associated problems like high phenolic exudation and presence of endophytic fungi. However, Maju and Soniya (2012) reports a successful rapid multiplication and transformation from seedling explants and Jiby and Bhat (2011) reports successful regeneration of transformed embryogenic mass from mature seeds. These protocols have limited utility due to lack of genetic identity. Hence a protocol based on *in vitro* derived juvenile explants will be a better alternative. Thus our objective was to standardize an appropriate protocol for transformation and regeneration involving juvenile tissues of somatic embryo derived plants. Somatic embryogenesis protocol developed here may be also utilized for large-scale clonal propagation and synthetic seed production. The paper thus reports an improved method for successful development of *osmotin* carrying transgenic black pepper and their enhanced tolerance to infection by *P. capsici* as evident from detatched leaf assay as well as stem/leaf inoculation methods.

Materials and methods

Plant materials

The released varieties of black pepper, *viz.* Subhakara and Panniyur–1 conserved at the experimental farm, Peruvannamuzhi (11°34'N, 75°48'E and 60 m MSL) were used in the experiments. Embryonic axis, leaves and nodal segments were used as explants for micropropagation. Surface sterilization was done using mercuric chloride (0.1%) for 2 minutes under aseptic conditions. Initially explants were cultured on Murashige and Skoog (MS) medium containing 0.5 mg l^{-1} kinetin and on establishment were transferred to woody plant media (WPM) with 3 mg l^{-1} benzyl adenine (BA) and 1 mg l^{-1} kinetin for multiplication. Explants were collected from these established cultures and transferred to any MS/WPM basal medium supplemented with BA (0.05-1 mg l^{-1}) and thidiazuron (TDZ) (0.05-1 mg l^{-1})

for regeneration. Rooting was induced in presence of indole butryic acid (IBA) (0.25-0.5 mg l^{-1}).

Somatic embryogenesis and regeneration

Very young leaves (preferably the freshly opened leaf of 1-2 cm size) from established aseptic cultures were selected for inducing somatic embryos. Leaf explants were cultured in the same medium and kept in dark for 15-20 days for initiating somatic embryogenesis. The petiole of the leaf was kept intact and in contact with the medium. Observations were made periodically. The regenerated calli/somatic embryos were very carefully transferred to medium of same composition. The somatic embryos generated by budding were carefully transferred to growth regulator- free liquid medium and agitated on orbital shaker at 100 rpm for proliferation. All the operations were done in dark to enhance adventitive emrbyogenesis and elongation and maturation of embryos. The embryos were separated based on their maturity and cultured separately for development of plantlets.

Plant expression vector, transformation and development of putative transgenics

Agrobacterium tumefaciens strain pGV 2260 carrying a recombinant plasmid pBinAR having a selectable marker, kanamycin resistance gene (*npt II*) and the gene '*osmotin*' under the control of CaMV 35S promoter and nopaline synthase (NOS) terminator was used as the vector system for transformation (Fig. 1). Selection medium comprised of 50 µg ml^{-1} kanamycin + 250 µg ml^{-1} cefotaxime in WPM. YEM medium with 100 µg ml^{-1} rifampicin, 100 µg ml^{-1} carbenicillin, 50 µg ml^{-1} kanamycin was used for bacterial inoculation and grown overnight at 28 °C.

Shoot tips and leaf explants from aseptically grown sterile plants derived from somatic embryos were most commonly used. Leaves were cut into small pieces in WPM eliminating the midrib portion and placed in a Petri dish (90 mm) and 10 ml of the overnight bacterial culture diluted five times with fresh YEM medium was used. Explants were thoroughly mixed with the bacterial suspension for 15 minutes by gentle swirling and blot dried with sterile tissue paper. The explants were placed upside down in a petriplate containing WPM medium and sealed with parafilm and kept for co-cultivation in dark for two days at 28 °C in a culture room. Explants were then washed with liquid MS medium containing 250 µg ml^{-1} cefotaxime and blot dried with sterilized tissue papers and incubated for 2 to 4 days on a nonselective medium WPM (BAP 3 mg l^{-1}, kinetin 1 mg l^{-1} + 3% (w/v) sucrose) containing 250 µg ml^{-1} cefotaxime. The tissues were then transferred to selective WPM containing 50 µg ml^{-1} Kanamycin and 250 µg ml^{-1} cefotaxime and incubated in the culture room for about three weeks, till many green shoots appeared along the cut edges. Green shoots were maintained on WPM medium with antibiotics and transferred to hormone- free WPM media for inducing rooting. Control explants were kept without co cultivation on selective medium.

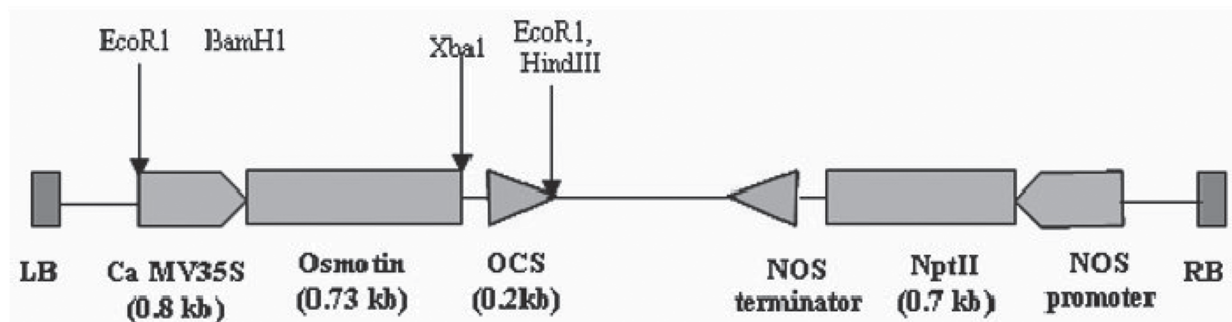

Fig.1. Schematic representation of binary vector Bin AR used in transformation of black pepper. The T-DNA region of the binary vector showing the assembly of osmotin expression cassette (CaMV 35 S P: osmotin: OCS) and kanamycin expression cassette (Nos P: *npt II*: Nos Ter). *CaMV 35S P CaMV 35S* promoter; *OCS* Octopine synthase gene terminator; *Nos P* Nopaline synthase promoter; *Nos Ter* Nopaline synthase terminator; *npt II* Neomycin phosphor transferase enzyme (Husaini and Abdin, 2008)

Molecular detection of transgenic plants by polymerase chain reaction

Genomic DNA was extracted from young leaf tissues of five putative transformants as per the modified CTAB protocol (Ausubel *et al*., 1994). An amount of about 0.2 g leaf tissues was ground in liquid nitrogen and were transferred to a 1.5 ml centrifuge tube containing 750 µl preheated CTAB extraction buffer (0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA and 2% CTAB). Samples were mixed thoroughly by gentle shaking and kept for incubation at 65 ^{o}C for 30 minutes. After chloroform-isoamyl treatment, DNA was precipitated with cold isopropanol and resuspended in TE buffer. DNA was purified by treating with RNase and extracting once with phenol/chloroform followed with chloroform/isoamyl alcohol. DNA was reprecipitated with ethanol and suspended in TE buffer. DNA concentration was estimated by comparison with Lambda DNA standard in a 0.8 per cent agarose gel and documented.

The presence of *osmotin* and *npt II* genes in the genomes of the transformants was studied by the PCR amplification using *osmotin* specific primer (TCCTTGCCTTGGTGACTT-3' and 5'-CAACTG TCCGTACACCGT-3') and *npt II* specific primer (GAGGCTATTCGGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3'). Each PCR reaction consisted of 40 ng template DNA, 1.0 Unit Taq DNA polymerase (Finnzymes), 10X Assay buffer (50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, and 100 µl ml^{-1} of gelatin), 150 µM dNTP's, 0.1 µM each of osmotin/ *npt II* specific primers. The amplifications were performed with PCR machine (Applied Biosystems) using the following thermocycling conditions. Initial denaturation-of one cycle at 94 ^{o}C for 3 minutes, 33 cycles of 94 ^{o}C for 1 minute, 58 ^{o}C for 1 min, 72 ^{o}C for 1 minute followed by a final extension at 72 ^{o}C for 5 minutes. Products were analysed on 2.0 per cent agarose gel for the presence of expected band and documented.

Pathogen inoculation and screening of putative transgenic plants

Two methods of screening were used *viz.,* a) inoculation of detatched leaves from transgenic plants and b) root/stem/leaf inoculation of intact hardened plants maintained in the green house. A virulent isolate of *P. capsici* (02-01) obtained from National Repository of *Phytophthora,* Indian Institute of Spices Research, Kozhikode was used for screening. In the first method, healthy leaves were detached from putative transgenic plants and kept in the humid chamber. The preserved isolate was cultured in carrot agar media for 48 hours and a 5 mm disc from the margin was cut and kept on the lower surface of the leaf. The leaf with the 5 mm disc with moisture content was kept for incubation for 72 hours and the observations were made at 24 hours intervals.

The four putative transgenics *viz.* TGP 1, 3, 7 and 10 maintained in the green house were subjected to stem, leaf and root inoculation using *P. capsici.* In case of intact plants, rooted cuttings of 3-4 leaf stage raised in sterile potting mixture (1:1:1) in polythene bags of size 21 cm x 15 cm were used for screening and experiment was conducted in three replications of five plants each. Inoculum plugs of 3 mm were placed on the lower surface of third or fourth leaf from the top of the plant. A moist cotton strip was placed over the inoculum plug and was secured using cello tape. Observations on leaf lesion diameter were recorded at 72, 120 and 192 hours.

For stem inoculation, a slight pinprick injury was made on the third internode on the stem from the top of the plants and a mycelial plug (5 mm size) of *P. capsici* cut from the edge of a 72 hours old actively growing mycelia was placed over it and covered with wet cotton pad and tied with polythene strip in order to keep the inoculum continuously wet. The inoculated plants were incubated for 72 h at 24-25 °C at a relative humidity of 75-90 per cent. After 72 hours, the external stem lesion diameter were measured and scored on a 0-4 scale.

Root inoculation was perfomed on a set of three month old rooted cuttings in polythene bags. A 72 hour old culture of *P. capsici* in the form of mycelial discs was used. Five numbers of 10 mm mycelial plugs were incorporated into the root zone of five rooted plants and observations were recorded on infection and mortality. Root/collar infection is manifested as decay of the collar portion, which extends upwards resulting in the total collapse of the plant. The mortality of the plant was taken as the measure of disease severity.

Results and discussion

Regeneration from tissues and somatic embryogenesis

Plants could be successfully regenerated from tender leaf, shoot tip and hypocotyl explants of both mature and juvenile tissues using MS/WPM supplemented with BA (0.05-1 mg l^{-1}) and TDZ (0.05-1 mg l^{-1}). Somatic embryogenesis was also induced successfully from tender leaf explants of *in vitro* established mature tissues using MS/WPM supplemented with BA (0.05-1 mg l^{-1}) and TDZ (0.05-1 mg l^{-1}) (Fig. 2 a&b).

Development of an efficient protocol for black pepper transformation and its subsequent regeneration is a prerequisite for production of transgenic plants. Plants could be successfully regenerated from callus cultures of many *Piper* species. Protocols for plant regeneration through organogenesis as well as somatic embryogenesis in black pepper were standardized from shoot tip and leaf tissues with or without intervening callus phase (Babu *et al.*, 1997) and from tissues of germinating seeds (Nair and Gupta, 2003; Jiby and Bhat, 2011). However, this is the first report of somatic embryogenesis from leaf tissues of mature black pepper plants.

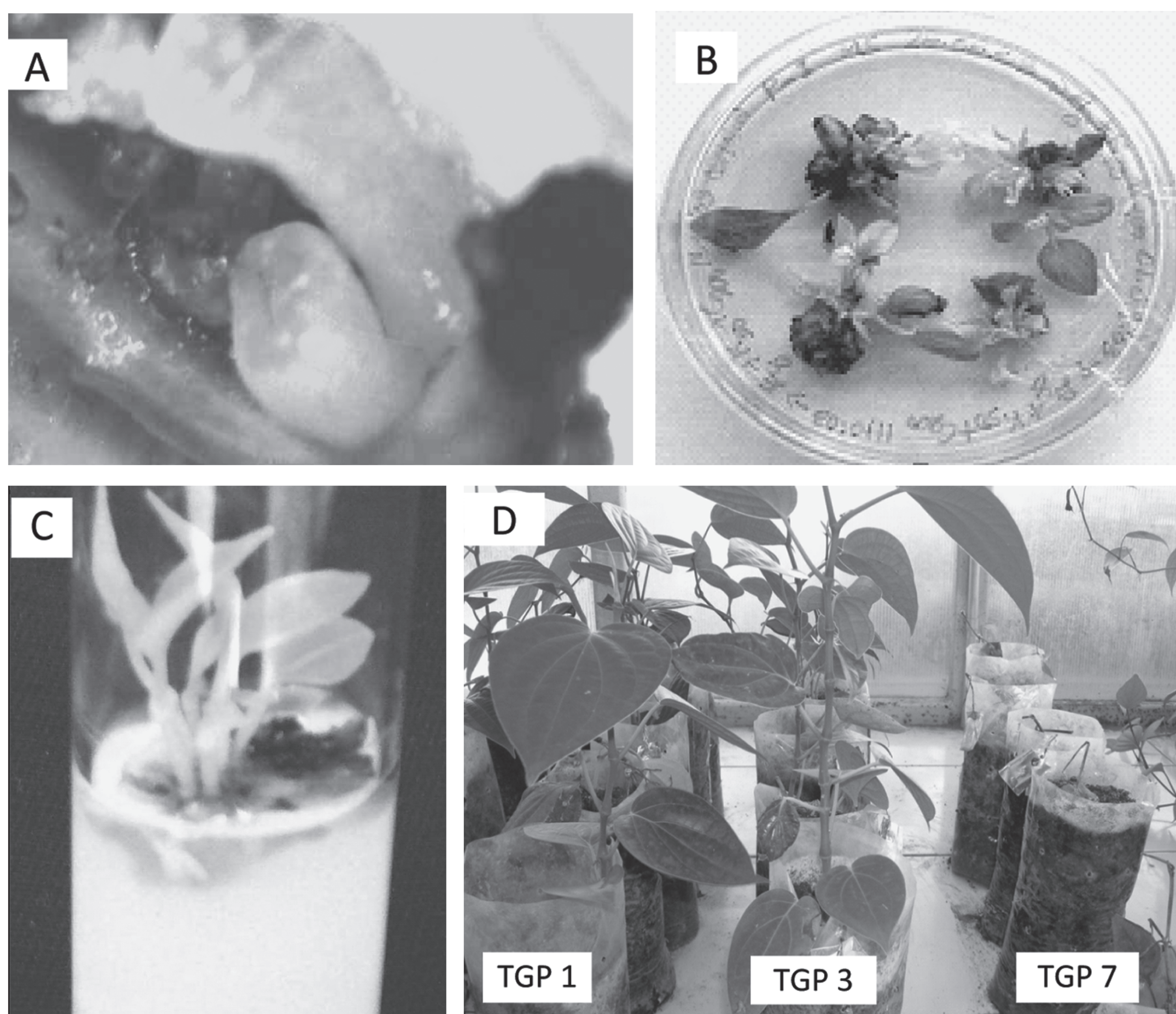

Fig. 2. Somatic embryogenesis, regeneration and screening of black pepper transgenic plants carrying osmotin. A) Induction of somatic embryos from leaf tissues; B,C) Plant regeneration from *Agrobacterium* co-cultivated cultures; D) Hardened plants of TGP 1 & 3 survived infection, while TGP7 died

Transformation and generation of transgenic plants

For effective shoot regeneration after transformation, the procedures for preconditioning, co-cultivation and delay selection were optimised. Two days of preconditioning followed by two days of co-cultivation gave the best response. When co-cultivation was extended beyond two days, tissue necrosis and subsequent cell death due to infection of the explants occurred. Shoot regeneration frequency has decreased drastically after successive transfer through selection medium. However a few putative transgenics were successfully regenerated from mature leaf explants and hypocotyls of *in vitro* grown seedlings treated with *Agrobacterium* containing *osmotin* (Fig. 2c).

Preliminary reports are available on *Agrobacterium* mediated gene transfer system in *P. nigrum* (Sasikumar and Veluthambi, 1996). They reported that kanamycin at 50 µg ml^{-1} completely inhibited callus formation and growth and hence is the minimum concentration needed to select transformed tissues of black pepper. They have also obtained primary transformants for kanamycin resistance in the cotyledons using *A. tumefaciens* binary vector strains LBA 4404 and EHA 105. However, they failed to induce regeneration from the transformed tissue. Sim *et al*. (1998) and Jiby and Bhat (2011) also report *Agrobacterium* mediated transfer of GUS reporter gene to black pepper. However, this is the first report on development of transgenic plants from juvenile tissues derived from somatic embryos of mature leaf explants.

Molecular analysis of transgenic plants

The presence of *osmotin* and *npt II* genes in the genomes of the transformants was studied by the PCR amplification using gene specific primers. Products were analysed on 2.0 per cent agarose gel for the presence of expected band. The presence of *npt II* gene in all the transgenics was confirmed by the presence of specific 700 bp band (Fig. 3a). The 550 bp product specific for *osmotin* was also amplified from a few putative transformants, indicating the transgenic nature (Fig. 3b). There were no comparable bands of that particular size in non transgenics.

Disease evaluation of transgenic plants

Detatched leaf assay

The detatched transgenic leaves showed different degrees of *P. capsici* infection when observed at 48 and 72 hours after inoculation. There was delayed infection and decreased rate of disease spread in all the transgenics compared to the control indicating differential integration and expression of osmotin. One of the inoculated plants did not take up infection even after 72 hours indicating disease tolerance.

Stem and leaf inoculation

Stem and leaf inoculated plants of TGP-7 died by the 10th day of inoculation (Fig. 2d) whereas, TGP-1, 3 and 10, took up infection but the lesion extended only till the next node from where an abscission layer was formed and the infected portion got detached from the plant and the plants survived.

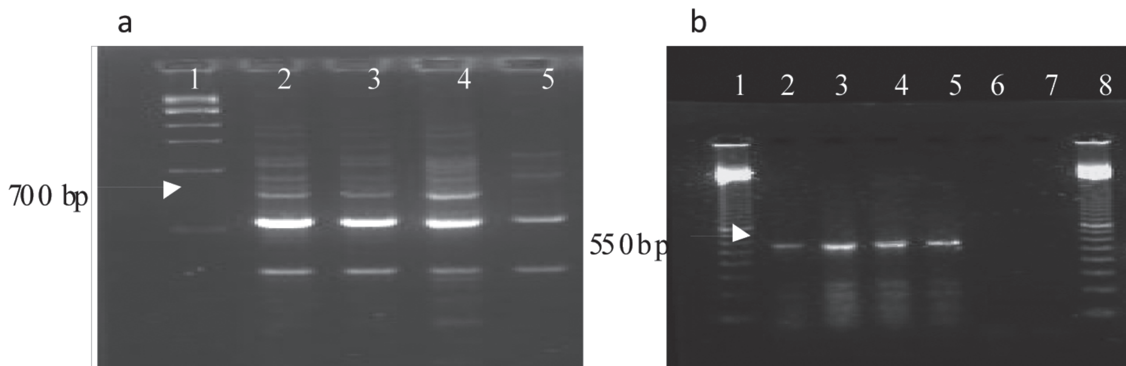

Fig. 3. PCR amplification of *npt II* and *osmotin* in the putative transgenics. a. lanes 1= 1 Kb Ladder, 2-4= transgenic plants, 5= control, b. lanes 1&8= 100 bp ladder, 2-5= transgenic plants, 6&7= control

Table 1. Response of transgenic plants to infection by *P. capsici* and screening site mean lesion scores in five replicas

Transgenic plant	Leaf lesion (mm) (0-4 scale)*			Stem lesion (mm) (0-4 scale)*			Root collar infection (mm) (0-4 scale) *			Present status
	3d	5d	8d	3d	5d	8d	7d	10d	15d	
TGP 1	18.85 (4)	29.0 (4)	Leaf fallen	31.75 (4)	52.75/ 57.0 (4)	Fallen	Initiation of infection	35.0 (4)	died	Both stem and leaf inoculated plants survived, lesion not extended
TGP 3	15.55 (4)	22.10 (4)	Leaf fallen	23.50 (4)	37.50/ (4) 46.33	46.33 (4) Lesion not extended	Died after 30 days			Both stem and leaf inoculated plants survived, lesion not extended
TGP 7	12.90 (3)	20.80 (4)	Leaf fallen and lesion extended to stem	35.80 (4)	55.75/ 85 (4)	Lesion extended downwards	25 (4)	died	died	All plants died on the 10th day
TGP 10	19.25 (4)	32.45 (4)	Leaf fallen	48.20 (4)	58.0/ 68.5 (4)	Lesion not extended	Initiation of infection	48.33 (4)	died	Both stem and leaf inoculated plants survived, root inoculated plants died
Control Sreekara	17.20 (4)	33.0 (4)	-	31.40 (4)	55/60 (4)	Lesion extended bothways	Initiation of infection	170.0 (4)	died	All plants died on the 15th day

*Lesion length (0-4 scale): 0 for no lesion, 1 for 1-5 mm lesion, 2 for 6-10 mm, 3 for 11-15 mm and 4 for >15 mm lesion
Values in the paranthesis indicate the score; d - days

However, all the plants were found susceptible to root infection (Table 1). It can be inferred that *osmotin* carrying transgenics showed a definite advantage when it came to survival after infection by *P. capsici.* Higher plants respond to various physical, chemical and biological stresses in a variety of ways. PR-proteins were found to be induced by bacterial and fungal infections in a number of plant species (Redolfi, 1983). Plants have developed a variety of defense mechanisms that enable them to survive severe environmental stress and pathogen attack. Rapid activation of stress-inducible genes and *de novo* synthesis of a large number of 'stress proteins' have been proposed to be at least part of a plant defense mechanism. Osmotin is a member of the PR-5 family that was originally identified as the predominant protein that accumulated in tobacco cells as a function of osmotic adaptation (Singh *et al.*, 1987). Subsequently, osmotin and other osmotin-like proteins were shown to have antifungal activity *in vitro* against a broad range of fungi, including several plant pathogens (Yun *et al.*, 1997).

Osmotin is reported to induce host plant resistance to various biotic stresses caused by fungal pathogens. Induction of host plant resistance against *Phytophthora* species is reported in potato, tomato and tobacco (Yun *et al.*, 1997). Although little progress has been made in elucidating the function of osmotin in osmotic stress, there is accumulating evidence that osmotin confer antifungal activity in plants (Liu *et al.*, 1994). The fungal growth inhibition by osmotin is correlated with plasma membrane permeabilization and dissipation of the membrane potential by Abad *et al.* (1996) who suggest a physical interaction between PR 5 proteins and the plasma membrane of sensitive fungi. Osmotin is also reported to interfere with pathogen signal transduction pathway to enhance fungal cell susceptibility (Yun *et al.*, 1997). There is

accumulating evidence that osmotin and osmotin-like proteins cause lysis of sporangia and growth inhibition of *P. infestans* (Woloshuk *et al*., 1991).

Thus delayed infection and decreased disease impact in transgenics might be attributed to antifungal activity (Mondal *et al*., 2003). The significance of the present study is to infer that osmotin gene can confer fairly good resistance to *Phytophthora*. The study also showed that expression of resistance differs between roots and intact transgenic plants. It is thus crucial to include root resistance properties into the characterization of transgenic plants (Germundsson *et al*., 2002). In the absence of any other plant genes in the black pepper germplasm to induce resistance to *Phytophthora*, this alternative strategy may be adopted for developing transgenics which over-express pathogen related proteins that impart tolerance to *P. capsici* infection. These transgenics may be also utilized as parents in traditional breeding programmes for *Phytophthora* resistance.

Acknowledgements

The Authors are thankful to Dr. K.C. Bansal, National Research Centre for Plant Biotechnology, IARI Campus, New Delhi for sparing the Osmotin construct for the experiments. The authors gratefully acknowledge the financial support received from the Department of biotechnology, New Delhi, facilities and guidance received from Director, Indian Institute of Spices research, Kozhikode.

References

Abad, L.R., D'urzo, M.P., Liu, D., Narasimhan, M.L., Reuveni, M., Zhu, J.K., Niu, X., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. *Plant Science* **118**: 11-23.

Asha, S. and Rajendran, P.C. 2010. Putative transgenic plants through *in planta* transformation against *Phytophthora* foot rot in black pepper (*Piper nigrum* L.). *Asian Journal of Bio Science* **4**: 135-141.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1994. *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley- Interscience, New York.

Babu, K.N., Ravindran, P.N. and Peter, K.V. 1997. Protocols for micropropagation of spices and aromatic crops. Indian Institute of Spices Research, Kozhikode, Kerala. 35p.

Bhai, R.S., Eapen, S.J., Anandaraj, M. and Saji, K.V. 2010. Identification of *Phytophthora* and nematode-resistant source -from open pollinated progenies of black pepper (*Piper nigrum* L.) using a modified protocol. *The Indian Journal of Agricultural Sciences* **80**: 893-897.

Biju, J., Dominic, J. and Philip, V.J. 1996. Plant regeneration from somatic embryos in black pepper. *Plant Cell, Tissue and Organ Culture* **47**: 87-90.

El-Kereamy, A., El-Sharkawy, I., Ramamoorthy, R., Taheri, A., Errampalli, D., Kumar, P. and Jayasankar, S. 2011. *Prunus domestica* Pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. *PLoS ONE*, **6**(3): e17973.

Germundsson, A., Sandgren, M., Barker, H., Savenkov, E.I. and Valkonen, J.P.T. 2002. Initial infection of roots and leaves reveals different resistance phenotypes associated with coat protein gene-mediated resistance to Potato mop-top virus. *Journal of General Virology* **83**: 1201-1209.

Grover, A. and Gowthaman, R. 2003. Strategies for development of fungus resistant transgenic plants. *Current Science* **84**: 330-340.

Jongedijk, E., Tigelaar, H., Van roekel, J.S.C., Bres-vloemans, S.A., Dekker, I., Van Den Elzen, P.J.M., Cornelissen, B.J.C. and Melchers, L.S. 1995. Synergistic activity of β 1,3 glucanases and chitinases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85**: 173-180.

Jiby, M.V and Bhat, A.I. 2011. An efficient *Agrobacterium* mediated transformation protocol for black pepper (*Piper nigrum* L.) using embryogenic mass as explant. *Journal of Crop Science and Biotechnology* **14**: 247-254.

Li, R., Wu, N., Fan, Y. and Song, B. 1999. Transgenic potato plants expressing osmotin gene inhibits fungal development in inoculated leaves. *Chinese Journal of Biotechnology* **15**: 71-75.

Liu, D., Raghothama, P.M., Hasegawa, P.M. and Bressan, R.A. 1994. Osmotin overexpression in potato delays development of disease symptoms. *Proceedings of National Academy of Sciences* **91**: 1888-1892.

Maju, T.T. and Soniya, E.V. 2012. *In vitro* regeneration system for multiplication and transformation in *Piper nigrum* L. *International Journal of Medicinal and Aromatic Plants* **2**: 178-184.

Mani, T., Sivakumar, K.C. and Manjula, S. 2012. Expression and functional analysis of two osmotin (PR 5) isoforms with differential antifungal activity from *Piper colubrinum*: Prediction of structure function relationship by bioinformatics approach. *Molecular Biotechnology* **52**: 251-261.

Mondal, K.K., Chatterjee, S.C., Viswakerma, N., Bhattacharya, R.C. and Grover, A. 2003. Chitinase mediated inhibitory activity of *Brassica* transgenic and growth of *Alternaria brassicae*. *Current Microbiology* **47**: 171-173.

Nair, R.R. and Gupta, S.D. 2003. Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.): Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos. *Journal of Horticultural Science and Biotechnology* **78**: 416-421.

Redolfi, P. 1983. Occurrence of pathogenesis-related (b) and similar proteins in different plant species. *Netherlands Journal of Plant Pathology* **89**: 245-252.

Sasikumar, B. and Veluthambi, K. 1996. Transformation of black pepper (*Piper nigrum* L.) using *Agrobacterium* Ti plasmid based vectors. *Indian Perfumer* **40**: 13-16.

Sim, S.L., Jafar, R., Power, J.B. and Davey, M.R. 1998. Development of an *Agrobacterium* mediated transformation system for black pepper (*Piper nigrum* L.). *Acta Horticulturae* **461**: 349-354.

Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa A.K., Buckel S., Hermodson, M.A., Pfankoch, E.D., Regnier, F.E. and Bressan, R.A. 1987. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. *Plant Physiology* **85**: 529-536.

Stephen, R.J., Anandaraj, M. and Sarma, Y.R. 2001. Induction of PR-proteins and defense related enzymes in black pepper due to inoculation with *Phytophthora capsici*. *Indian Phytopathology* **54**: 23-28.

Vanaja, T., Neema, V.P., Mammootty, K.P. and Rajeshkumar, R. 2008. Development of a promising interspecific hybrid in black pepper (*Piper nigrum* L.) for *Phytophthora* foot rot resistance. *Euphytica* **161**: 437-445.

Woloshuk, C.P., Meulenhoff, J.S., Sela-buurlage, M., Van den elzen, P.J. and Cornelissen, B.J. 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *The Plant Cell* **3**: 619-628.

Yun, D.J., Bressan, R.A. and Hasegawa, P.M. 1997. Plant anti-fungal proteins, In: *Plant Breeding Reviews*, (Eds.) Janick, J, John Wiley & Sons, New York. pp. 39-88.