Archives of Phytopathology and Plant Protection
February 2006; 39(1): 33–37

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Rhizospheric *Pseudomonas fluorescens* enhances piperine production in *Piper nigrum*, a possible means of biochemical defence against *Phytophthora capsici*

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(Received 29 November 2004)

Abstract
Black pepper is an important export-oriented spice crop. Foot rot caused by *P. capsici* is a very serious disease, which cause serious economic loss to the farmers. Biological control is the viable strategy for sustainable disease management. Efficient strains of *P. fluorescens* reduced the foliar infection caused by *P. capsici* significantly. It has been observed that the level of piperine, the pungent principle in black pepper, is increased to significant levels upon root bacterization of the black pepper vines. In addition to it, piperine (Sigma) inhibited the mycelial growth of *P. capsici, in vitro,* demonstrating the direct fungicidal activity of this alkaloid. An increase in the quantity of piperine is supposed to contribute to the overall host defence mechanism of the plant. The paper describes for the first time, the rhizobacteria-mediated induction of piperine in black pepper.

Keywords: *Black pepper, piperine,* P. fluorescens, *host defence*

Introduction

Black pepper (*Piper nigrum* L.) known as 'King of Spices' and 'Black gold' is the most important and most widely used spice in the world, occupying a position that is supreme and unique. The spicy flavour of peppers comes from the alkaloid, piperine. The piperine's tangy and spicy taste is the perfect additive for most foods. Piperine is the trans-trans isomer of 1-piperoyl piperidine and represents 90–95% of the total pungency of black pepper (Anil et al. 1994).

The present study dealt with the rhizobacteria-mediated up-regulation of piperine and the inhibitory effect of piperine on the disease (foot rot) causing fungi (*Phytophthora capsici*) in black pepper. *Phytophthora*-foot rot of black pepper is the major production constraint in almost all the major pepper-growing countries (Sarma 2003). This soil-borne oomycetous fungus affects all parts of the plant. Disease occurs in two phases, viz., aerial

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ISSN 0323-5408 print/ISSN 1477-2906 online
DOI: 10.1080/03235400500180677

and soil. Integrated Disease Management with a major emphasis on biocontrol has been evolved to combat this malady. The ability of rhizosphere-associated bacteria to inhibit the growth of plant pathogenic fungi has generated increased interest in their use as crop protectants.

Systemic resistance triggered in the plant by rhizobacteria is referred to as rhizobacterial-mediated induced systemic resistance (ISR) (van Loon et al. 1998). *Pseudomonas fluorescence* could act as strong elicitors of plant defence reaction (M'Piga et al. 1997). ISR is brought about by Plant Growth Promoting Rhizobacteria (PGPR) by changing the physiological and biochemical reactions of the host leading to the synthesis of defence chemical against the challenge pathogen (Benhamou et al. 1996). Activation of defence genes by prior application with PGPR against a challenging pathogen is a novel strategy in plant protection. The increased activity of the above substances in the PGPR-treated plants may play either a direct or an indirect role in the suppression of pathogen development in the host ultimately protecting the plants from pathogenic micro-organism. However information is scanty about the induction of various alkaloids involved in phenyl propanoid pathway, due to *Pseudomonas* treatment.

Material and methods

The bacterial strains

Out of over 1,000 strains of rhizobacteria screened at the Indian Institute of Spices Research (IISR), few strains were short-listed based on their efficiency in root rot suppression in black pepper, growth promotion and induction of systemic resistance. The efficient ones were tested *in planta* for their efficiency in enhancing the levels of piperine in the plant. The five strains of *Pseudomonas fluorescens* used were IISR-6, IISR-8, IISR-11, IISR-13 and IISR-51.

Bacterization and estimation of piperine content

Black pepper plants were root-bacterized with the five strains of *P. fluorescens* as separate treatments. Suitable replications were maintained. Upon two months of growth, leaves from each plant in all treatments were collected. The leaves of each treatment were pooled together, cut into small pieces, 1 g of it were ground in mortar and pestle and refluxed in a round bottom flask for 30 min after adding 25 ml of distilled alcohol. The extract was collected in a 50 ml standard flask after passing through a layer of cotton. The residue was washed three times with alcohol, pooled and added to the standard flask and the volume was made up to 50 ml with alcohol. Ten ml of it was filtered through a Whatman filter paper and taken in a 25 ml standard flask. It was made up to 25 ml with the mobile phase, prepared earlier. The mobile phase was prepared with acetic acid (1%) and acetonitrile (both HPLC grade) in 1:1 proportion. This sample (25 μl) was injected to the HPLC. From the area of peaks falling in the range for piperine, the % (mg) of piperine was calculated.

Disease suppressive activities

The root-bacterized plants, after two months, were sprayed with a suspension of *P. capsici* zoospores (log-2 spores/ml) and subsequently observed for lesion development in the leaves, visual scoring was made and the data analysed.

Fungicidal activity of piperine

The direct fungicidal potential of piperine was tested with a plate assay. In order to study the lethal effect of piperine on *P. capsici*, concentrations of 250 and 500 ppm of pure Piperine (Sigma) were prepared in 95% ethanol. Sterile potato dextrose agar (PDA) plates were prepared and a 2 mm disk of *P. capsici* was inoculated at the centre of the plate. Wells were made in the agar; 2 cm away from the disk using cork borer and piperine stock solution was added in the wells. A well was maintained for ethanol as negative check. The plates were incubated at 25°C for 48 h and looked for inhibition of *Phytophthora* mycelium.

Results and discussion

An increase of 48% was recorded in the synthesis of piperine in the *Pseudomonas*-treated plants. The highest induction was found with the strain, IISR-6 treated plants (see Figure 1).

The increased quantity of piperine in leaves is supposed to play a role in the resistance mechanism of black pepper against disease either directly or indirectly, as there was a reduction in leaf lesion in the root-bacterized plants after challenge inoculation with *P. capsici*. The lowest lesion index was observed with the treatment with the strain, IISR-6 (see Table I).

There have been various reports on the induction of various alkaloids and phenolic compounds in agricultural crops upon treatment with rhizobacteria. A massive accumulation of phytoalexin (van Peer et al. 1991), phenolic compounds (Diby Paul 2004; M'Piga et al. 1997) increase in the activities of PR proteins (Maurhofer et al. 1994), peroxidase (Albert & Anderson 1987; Zdor & Anderson 1992) increase in the levels of mRNAs encoding phenyl alanine ammonia lyase (PAL) and enhanced lignification (Anderson & Guerra 1985) have been reported in plants following treatments with PGPR strains.

It was observed that the tested concentrations of 250 ppm and 500 ppm of piperine inhibited the mycelial growth of *P. capsici* (see Figure 2). Piperine has been reported to have many pharmacological and antimicrobial effects. Hairselstrom et al. (1954) have reported the powerful fungistatic action of black pepper metabolites, piperine, apopiperine, ß-cinnamenyl acrolyl hydrazide, ß-cinnamenyl acrolyl piperide and isonicotinyl hydrazide on *Aspergillus*

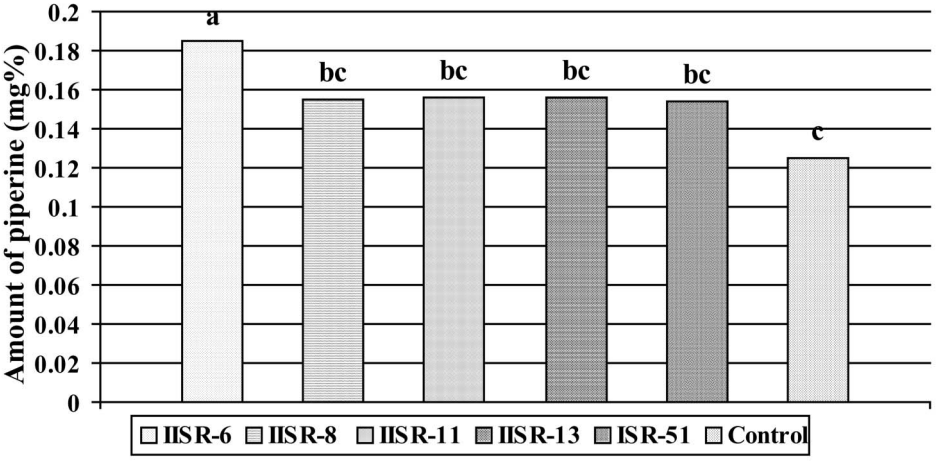

Figure 1. The amount of induced production of piperine in black pepper treated with *Pseudomonas* strains.

Table I. Leaf lesion index of black pepper after challenge inoculation.

S I No.	*P. fluorescens* strains	Lesion index
1	IISR-6	1. 113[f]
2	IISR-8	1.392[d]
3	IISR-11	1.634[c]
4	IISR-13	1.210[e]
5	IISR-51	2.100[b]
6	Control	3.129[a]

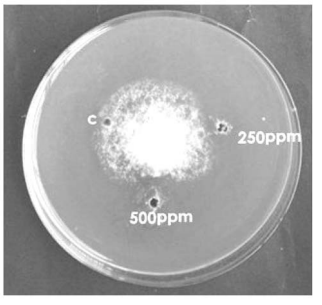

Figure 2. Lethal effect of piperine on *P. capsici.*

versicolor. Fungicidal activities of piperidine and tetrahydropyridines have also been reported (Mandal 1991).

The study demonstrates for the first time, the induced biosynthesis of piperine in black pepper vine due to the root-treatment of the vine with beneficial strains of *P. fluorescens.* The piperine could be acting as a biochemical defence in the plant against *P. capsici*. This attribute of the rhizobacteria is also useful for enhancing the quality of pepper since the major pungent principle in pepper is piperine. Studies on the bacterial determinants of piperine induction have to be undertaken in detail to understand this phenomenon better.

Acknowledgement

Support and help from the Director of IISR and Head, Division of Crop Protection and the financial assistance from the Department of Biotechnology, Government of India, are gratefully acknowledged.

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