Research Article

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Secondary metabolites in black pepper (*Piper nigrum*) and their effect on the foot-rot pathogen *Phytophthora capsici*

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Abstract

Most selections of black pepper are susceptible to the *Phytophthora* foot rot caused by *Phytophthora capsici*. Studies were undertaken to quantify the amount of secondary metabolites - piperine and oleoresins - in the vegetative plant parts of the susceptible selection KS27, tolerant selection P24 (both *P. nigrum*) and the resistant *Piper* species, *P. colubrinum*; and to study if they had any effect on the vegetative growth of the pathogen *in vitro*. The *P. nigrum* selections, KS27 and P24, especially the latter, had higher concentrations of piperine in the roots than the leaves or stems. Synthetic piperine was found to inhibit the vegetative growth of *P. capsici in vitro*, at concentrations of 0.4% and above. The oleoresin concentration was highest in the leaves, followed by the roots and least in the stems; the trend was the same in all three *Piper* plants with *P. colubrinum* recording the highest values. A significant inhibition in the growth of *P. capsici* was recorded when oleoresin from roots was incorporated in the media, less so in that containing leaf oleoresin, and an increase in growth in the presence of stem oleoresins. As the trend was the same in all three plants, no correlation could be drawn between inhibition of growth and susceptibility/tolerance/ resistance to *P. capsici*.

Keywords : Black pepper, *Piper nigrum*, *Piper colubrinum*, *Phytophthora capsici*, piperine, oleoresin.

Introduction

Spices may be defined as aromatic vegetable substances used for seasoning foods and consist of rhizomes, barks, leaves, fruits, seeds and other parts of plants. Black pepper (*Piper nigrum* L.), the 'King of spices', is the most important among the spices family. Dried berries of *P. nigrum* constitute the black pepper of commerce, the earliest known and the most widely used spice in the world. Black pepper is being decimated by the "*Phytophthora* foot-rot" caused by *Phytophthora capsici*. All parts of the vine are susceptible and the available variability for resistance to *P. capsici* appears to be little. However, a promisingly field tolerant, open pollinated seedling progeny, P24, has been identified (Sarma *et al.* 1994). In addition, a Brazilian species of *Piper*, *P. colubrinum*, has been found resistant to *P. capsici* (Albuquerque, 1968).

Black pepper contains starch, fibre and fat as major constituents, but more significant ones are the secondary metabolites - piperine and the volatile oils -which contribute to the pungency and aroma of the spice. Secondary metabolites, unlike the primary metabolites, have no generally recognized roles in the process of assimilation, respiration, transport and differentiation. They also differ from primary metabolites in having a restricted distribution and are typically found in only one plant species or taxonomically related group of species. Plant secondary products are categorized according to their mode of biosynthesis as terpenes, phenolic compounds and alkaloids.

Piperine, the major pungency principle of pepper, is the trans-trans isomer of 1-piperoylpiperidine and represents 90-95% of the total pungency of pepper (Anil *et al.*, 1994). The geometrical isomers - chavicine,

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isochavicine, piperettine, piperanine and pyrroperine, occur as minor components (Ananthakrishna and Govindarajan, 1975). Piperine is reported to have many pharmacological and antimicrobial effects. Harselstrom *et al.* (1954) have reported the powerful fungistatic action of black pepper secondary metabolites, piperine, apopiperine, b-cinnamenyl acrolyl hydrazide, b-cinnamenyl acrolyl piperide and isonicotinyl hydrazide on *Aspergillus versicolor* at concentrations of 0.1%. Fungicidal activities of piperidine and tetrahydropyridines have also been reported (Mandal, 1991). Piperine has also been used as an insecticide and as a synergist for pyrethrin and allethrin, against houseflies, rice weevils and cowpea weevils (Sumathikutty *et al.* 1981). Semler and Gross (1988) analyzed the distribution of piperine content in *P. nigrum*, and found only traces in leaves and shoots of 2 to 4 year old plants, while more significant quantities (ca 0.03%) occurred in the roots. Mature, fully differentiated roots contained as high as 0.2% piperine, equaling amounts found in low quality berries.

Oleoresins are extremely concentrated products, obtained by solvent extraction, and give a closer approximation to the total flavor of spices. These are a blend of essential oil and resinous matter made up of pungent principles, colors, fixed oils, natural resins etc. They are heat stable and extremely powerful in their flavoring effect, e.g. 1 kg of pepper oleoresin equals 20 to 25 kg of ground pepper (Shankaracharya and Natarajan, 1975). Pepper yields 10-12% oleoresin of which 20-30% are volatile oils and 40-45% piperine. Anandaraj and Leela (1996), in their study on the toxic effects of plant extracts on *P. capsici*, found that *P. colubrinum* extracts inhibited mycelial growth and sporangial production.

The objective of this study was to estimate the concentration of the secondary metabolites, piperine and oleoresins, in the vegetative parts (leaf, stem and roots) of the susceptible (KS27) and tolerant (P24) selections of *P. nigrum* and also in the resistant species, *P. colubrinum*; and also to study their effect on the vegetative growth of *P. capsici*.

Materials and Methods

Quantification of piperine and oleoresin and their effect on P. capsici

Piperine

Plant material: Plants of KS27, P24 and *P. colubrinum*, grown from single-node cuttings in nursery bags of three months growth, were divided into leaves (all but the most tender and basal leaves were used), stem segments and roots. The samples, in three replications for each treatment, were analyzed, each replication being a composite of five plants.

Analytical procedures: Piperine was extracted and analyzed according to the procedure of Wood *et al.* (1988) using a Shimadzu reversed-phase HPLC (RP-HPLC). Samples were dried at 60^0C finely powdered in a Cyclotec Mill, and piperine extracted from 500 mg dry weight of sample by refluxing for 3 hrs in 96% v/v ethanol, excluding light. The samples were cooled, filtered through Millipore filter assembly of 0.45 µm pore size, and made to 25 ml volume with ethanol.

The mobile phase, acetonitrile-aqueous 1% acetic acid (1:1), was degassed before use. The column was a 5 mm Shim-pack CLS ODS-2 (250 x 4.6 mm ID), operated at a flow-rate of 1.5 ml min^{-1} using a Shimadzu LC-6A liquid chromatograph pump. Injection volume was 20 µl and run time was up to 28 min. Using an external standard of piperine (Sigma) of 0.05 and 0.1 mg ml^{-1} concentration in ethanol, the Rt was found to be ca. 7-9 min. The Shimadzu SPD-6AV, UV-visible spectrophotometric detector was set at the absorption maxima of piperine, ie., 343 nm. Quantification was achieved by comparison of piperine peak area of each sample with mean peak area for standard using the Shimadzu C-R4A chromatopac integrator. The percentage of piperine on dry weight basis was calculated using the formula,

$$A \times K \times [25/10] \times 100/m_x \times 100$$

where, A = area of piperine peak

m_x = mass of sample in mg

25/10 = dilution factor

K = response factor determined for standard

$K = m'/A$

where, $m' = m \times P_1$

m = mass of piperine in mg

P_1 = purity of standard determined spectrophotometrically

A = area of piperine peak in integrated units (standard)

m' = corrected mass of piperine in mg

Effect on *P. capsici*: *P. capsici* was cultured in 25 ml volumes of Bartnicki-Garcia's liquid media for vegetative growth (Bartnicki-Garcia, 1966), supplemented with a series of concentrations of synthetic piperine - 0.004, 0.04, 0.4 and 0.5% in 100 ml conical flasks. Two culture

discs (5 mm each) of the soil isolate of *P. capsici*, P128, were incubated in the above media for 10 days. All the treatments were at the optimal pH for the vegetative growth of *P. capsici*, ie., 6.5. The dry weight of mycelia was recorded in grams after incubation.

Oleoresin

Plant material: One year old plants grown in nursery bags were separated into leaves, stems and roots; three replications of each were taken, each replication being a composite of five plants.

Analytical procedures: Oleoresin was extracted according to the procedure described in the ASTA Analytical Methods (1968). Five grams of samples were dried at 60-80^0 C and powdered finely in a Cyclotec Mill. These powders were held in acetone, in a column, overnight. The oleoresin extracted by the acetone was drained off the column into pre-weighed beakers, acetone evaporated and beakers weighed gravimetrically. Oleoresin content was expressed as percentage on dry weight basis.

Effect on *P. capsici*: The oleoresin extracts obtained from the above were dissolved in 10 ml of absolute alcohol, and all replications of a treatment were pooled. Two discs of 5 mm each of the *P. capsici* isolate, P128, was incubated for 10 days in 25 ml volumes of Bartnicki-Garcia media for vegetative growth supplemented with oleoresin from the nine different sources - leaf, stem and root oleoresin from KS27, P24 and *P. colubrinum*. The concentration of oleoresin in this media was equivalent to that found in the plant parts, and a control without oleoresin and a blank with an equivalent volume of absolute ethanol were also maintained, all at the optimal pH of 6.5. The mycelial mat obtained at the end of 10 days of incubation was processed for recording the dry weight of mycelia in grams.

Results and Discussion

Concentration of Piperine

The concentration of piperine, in the leaf, stem and root parts of KS27, P24 and *P. colubrinum*, are presented in Table 1. Significant differences were observed both between the different plants and different parts of the plants in their piperine content. As can be seen from Fig. 1, piperine peak was not detectable in the leaves of KS27 and stem of *P. colubrinum*; and only traces in the roots of *P. colubrinum*. The prominent peaks of Rt 1-3 min, are probably those of chlorophyll, carotenoids and related pigments, also extractable with ethanol. *P. colubrinum* contained over 3 times more piperine in its leaves than P24; KS27 had 1.4 times more piperine in its stem than P24. However, the roots of P24 contained concentrations of over 2 times than those found in KS27. KS27 and P24 contained significantly more piperine in the roots than other plant parts: about 2.8 times more KS27 than its stem, and 8.3 times in P24. But in *P. colubrinum*, 14.8 times more piperine was detected in the leaves than in roots.

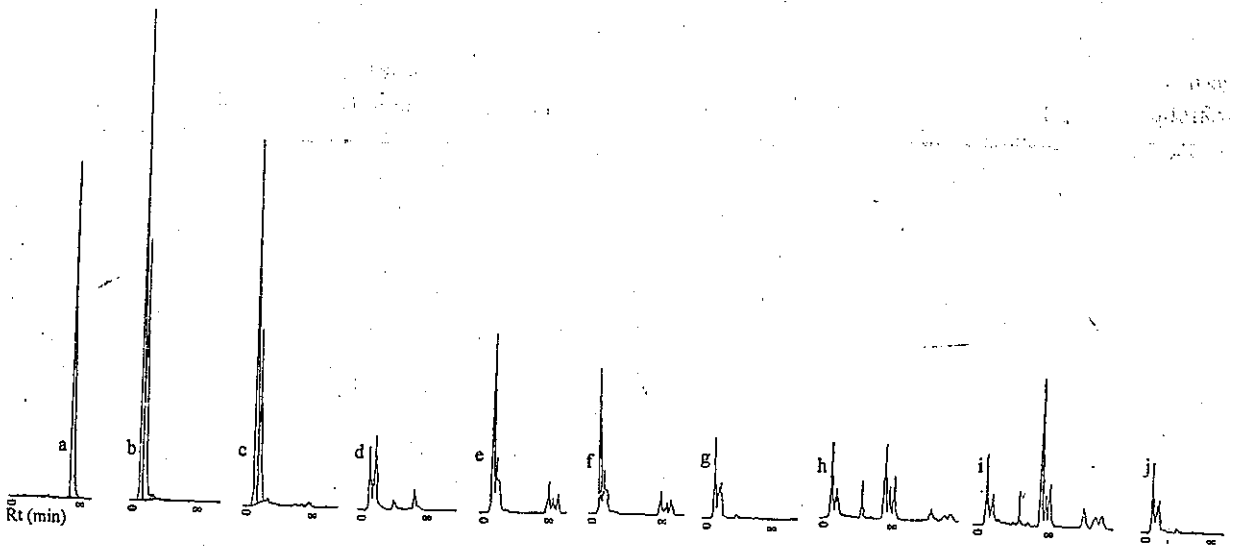

Fig 1. : RP-HPLC chromatogram of ethanolic extracts obtained from vegetative parts of KS27, P24 and *Piper colubrinum* plants, to determine piperine

Legend a - Piperine standard; b - KS27 leaf; c - P24 leaf; d - *P. colubrinum* leaf; e - KS27 stem; f - P24 stem; g - *P. colubrinum* stem; h - KS27 root; i - P24 root; j - *P. colubrinum* root

Table 1. Concentration of piperine in KS27, P24 and *P. colubrinum*

Plant	Piperine conc. (% in 100% dry weight) in			CD (5%) (Within a plant)
	Leaf	Stem	Root	
KS27	ND*	0.076	0.209	0.026
P24	0.019	0.053	0.440	0.044
P. colubrinum	0.059	ND*	0.004	0.001
CD (5%) (Between plants)	0.002	0.018	0.044	

*ND = Not Detectable

Effect of piperine on the vegetative growth of *P. capsici*

The effect of synthetic piperine, in a range of concentrations, including those found in the plant, were tried (Table 2). No significant difference was observed in the blank or treatments containing 0.004 and 0.04% piperine compared to control. However, a significant decrease in mycelial growth was observed in other treatments - 9.5% in the treatment containing 0.4% piperine, 18.2% with 0.5% piperine and over 52% at 5 and 10% piperine, compared to a control containing no piperine.

Table 2. Effect of concentrations of piperine on growth of *P. capsici*

Piperine (%)	Dry weight of mycelia (g)
Control (No piperine)	0.242
Blank (Ethanol)	0.241
0.004	0.244
0.04	0.243
0.4	0.219
0.5	0.198
5.0	0.114
10.0	0.115
CD (5%)	0.009

Most studies report piperine concentration in the berries for their obvious commercial importance. It varies between 2-8% depending on the maturity and cultivar (Sumathikutty *et al.* 1979; Gopalam and Ravindran, 1986). Quantitative analysis of piperine revealed that the roots of P24 and KS27 contained maximum amounts of piperine. P24, which is tolerant to *P. capsici*, has twice the concentration of the susceptible KS27; however, the resistant *Piper* species, *P. colubrinum*, had only traces. When the effect of synthetic piperine on the vegetative growth of *P. capsici, in vitro*, was studied, significant inhibition of growth was noticed only at concentrations of 0.4% and above. Such high concentrations are seen only in the roots of P24; whether this could be a factor in the tolerance of P24 to infection of the root as opposed to susceptibility to foliar infection (Shamina, 1997) is a moot point. But the fact remains that *P. colubrinum* with only traces of piperine in its roots is resistant and that the berries with far higher concentrations is susceptible. Thus, no relation appears to exist between piperine concentration and susceptibility, tolerance or resistance to *P. capsici*, though Harselstrom *et al.* (1954) have reported the powerful fungistatic action of black pepper piperine, on *Aspergillus niger.*

Concentration of oleoresin

The concentration of oleoresin in the leaf, stem and root parts of KS27, P24 and *P. colubrinum*, is given in Table 3. Highly significant difference was found between KS27, P24 and *P. colubrinum*, in their oleoresin concentrations in the leaf and root parts, the concentrations in the stem being on par. *P. colubrinum* had 71% more oleoresin than KS27 and 97% more than P24, in the leaves; and in the roots it was 78-80% more than either selections of *P. nigrum.*

Table 3. Concentration of oleoresin in KS27, P24 and *P. colubrinum*

Plant	Oleoresin conc. (g $100g^{-1}$ dry weight) in			CD (5%) (Within a plant)
	Leaf	Stem	Root	
KS27	5.008	1.428	3.138	0.357
P24	4.352	1.412	3.168	0.457
P. colubrinum	8.576	1.548	5.562	1.693
CD (5%) (Between plants)	1.463	0.418	1.018	

Within the plants, highly significant differences were found in the oleoresin concentration in the different parts. In KS27, it was least in the stems and maximum in the leaves, about 3.5 times more in the leaves, compared to stems and 1.6 times that found in roots. The trend was the same in P24 and *P. colubrinum*, though in the latter the leaves contained about 5.5 times the oleoresin found in stem and 1.5 times that of roots.

Effect of oleoresin on the vegetative growth of *P. capsici*

The effect of oleoresin, from the leaf, stem and root parts of KS27, P24 and *P. colubrinum*, on the vegetative growth of *P. capsici* was studied. A significant inhibition of the vegetative growth of *P. capsici* in media containing oleoresin from roots and less so in media with leaf oleoresin, was observed. Also a significant increase in growth was observed in media containing stem oleoresin. This trend was irrespective of the plant source, the difference being only in the magnitude as can be seen from Table 4.

Table 4. Effect of oleoresin extracted from different plant parts on growth of *P. capsici*

Plant	Dry mycelia (g) in oleoresin from (when grown in)			CD (5%) (within a plant)
	Leaf	Stem	Root	
KS27	0.152(-20.0)	0.253(+33.2)	0.101(46.8)	0.012
P24	0.157(-17.4)	0.216(+13.7)	0.109(-42.6)	0.009
P. colubrinum	0.124(-34.7)	0.222(+16.8)	0.089(-53.2)	0.015
CD (5%) (Between plants)	0.009	0.019	0.014	

Dry weight of mycelia (g) in Control = 0.190
Blank = 0.192

Figures in parentheses represent increase (+) or decrease (-) in %, in growth with respect to control.

P. colubrinum had much higher concentrations of oleoresin in its leaves and roots than the two selections of *P. nigrum* and it corresponded with a greater inhibition of *P. capsici* at the concentrations present in the plant. The root oleoresin mixtures had a significantly greater inhibitory effect than the leaf oleoresins, whereas the stem oleoresins supported growth. Again most reports of oleoresin content in black pepper are those of its berries (Shankaracharya and Natarajan, 1975). Essential oils of a number of plants have been reported as fungitoxic (Thompson, 1989; Garg and Siddiqui, 1992; Yegen *et al.* 1992), though no studies exist on black pepper. Though significant inhibition of *P. capsici* by the root and leaf oleoresins have been noticed in the present study, no correlation exists between this and susceptibility, tolerance or resistance to *P. capsici*.

The secondary metabolites, though considered as passive defense factors, did not play a positive role in defense response in this pathosystem. Of greater importance were the induction of defense related and scavenging enzymes and the membrane integrity of the system (Shamina, 1997).

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References

Albuquerque, F.C. 1968. *Piper colubrinum*, a grafting root stock for *Piper nigrum*, resistant to diseases caused by *Phytophthora palmivora* and *Fusarium solani* f. sp. *piperi*. *Pesqul Agropecu. Brasilia* 3: 141-145 (Spanish).

American Spice Trade Association (ASTA). 1968. Official analytical methods. II ed. American Spice Trade Association, NY. 38 p.

Anandaraj, M. and Leela, N.K. 1996. Toxic effects of some plant extracts on *Phytophthora capsici*, the foot rot pathogen of black pepper. *Indian Phytopathol.* 49 (2): 181-184.

Ananthakrishna, S.M. and Govindarajan, V.S. 1975. Evaluation of spices and oleoresins. V. Estimation of pungent principles of pepperl. *J Fd Sci Tech.* 12 (5): 253-256.

Anil, I, Roy, N.C., Raju, E.V., Joseph, M., Thomas, M. and Mathew, A.G. 1994. Pungent principles of pepper. *International Pepper News Bulletin.* 18 (2): 7-11.

Barnicki-Garcia, S. 1966. Chemistry of hyphal walls of *Phytophthora*. *J Gen Microbiol.* 42: 57-69.

Garg, S.C. and Siddiqui, N. 1992. Antifungal activity of some essential oil isolates. *Pharmazie* 47 (6): 467-468.

Gopalam, A. and Ravindran, P.N. 1986 Indexing quality parameters in black pepper cultivars. *Indian Spices* 22-23: 8-11.

Harselstrom, T., Dateo, G.P., Levinson, H.S., Stahl, W.H., Dewth, E.J. and Konigsbacher, K.S. 1954. Antioxidant activity and antagonism to microbial growth, piperine, isonicotynyl hydrazide and realted compounds. *Fd. Res.* 19 (4): 373.

Mandal, T.K. 1991. Synthesis and fungicidal activity of substituted 4-aminopiperidines and 4-aminotetrahydropyridines. *Khimiko-Farmatsevticheskii Zhurnal.* 25 (6): 28-33.

Sarma, Y. R., M. Anandaraj, and M. N. Venugopal 1994. Diseases of spice crops. In: *Advances in horticulture.* Vol. 10, Part 2. (Eds. K. L. Chadha and P. Rethinam). Malhotra Publishing House, New Delhi, India, pp. 1015-1058.

Semler, U. and Gross, G.G. 1988. Distribution of piperine in vegetative parts of *Piper nigrum*. *Phytochem.* 27 (5): 1566-1577.

Shamina, A. 1997. "Biochemical studies on host-pathogen interactions in black pepper (*Piper nigrum* L.) infected with *Phytophthora capsici*". Ph.D. thesis submitted to University of Calicut, Kerala, India.

Shankaracharya, N.B. and Natarajan, C.P. 1975. Technology of spices. *Arecanut & Spices Bulletin* 7 (2): 27-43.

Sumathikutty, M.A., Rajaram, K., Sankarikutty, B. and Mathew, A.G. 1979. Chemical composition of pepper grades and products. *J. Fd. Sci. & Tech.* 16: 249-252.

Sumathikutty, M.A., Rajaram, K., Sankarikutty, B., Narayanan, C.S. and Mathew, A.G. 1981. Piperine. *Lebensm-Wiss u Technol.* 14: 225.

Thompson, D.P. 1989. Fungitoxic activity of essential oil components on food storage fungi. *Mycologia.* 81 (1): 151.

Wood, A.B., Barrow, M.L. and James, D.J. 1988. Piperine determination in pepper (*Piper nigrum* L.) and its oleoresins - A Reversed-Phase High Performance Liquid Chromatographic method. *Flavour & Fragrance J.* 3 (2): 55-64.

Yegen, O., Berger, B. and Heitefuss, R. 1992. Studies on the fungitoxic effects of extracts of 6 selected palnts from Turkey on phytopathogenic fungi. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz.* 99 (4): 349-359.