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# Biochemical basis of resistance against root knot nematodes in chilli (*Capsicum annuum* L.)

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**ABSTRACT:** Chilli (*Capsicum annuum* L.) is an important and versatile vegetable cum spice crop. A study was conducted to understand the biochemical mechanism in two resistant advanced lines developed (ACRIL 90, ACRIL 70) along with two susceptible genotypes (Arka Mohini, Arka Suphal) , with and without incouation of RKN, *M. incognita*. The results showed that there was a significant increase in antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (PO), and polyphenol oxidase (PPO) in resistant genotypes compared to susceptible genotypes upon inoculation of nematodes. Resistant genotypes also recorded significant higher contents of lignin, phenol, and protein than the susceptible ones. Thus the current study proves that antioxidant enzymes, lignin and phenol contents play a significant role in inducing resistance in host plants against *M. incognita*.

**Keywords:** Capsicum, Root knot nematode, SOD, PO, PPO, lignin, phenols and protein

## INTRODUCTION

Chilli (*Capsicum annuum* L.) is a versatile plant that is cultivated as a vegetable and spice crop. The yield, quality and growth of plants are limited by many biotic and abiotic factors (Naresh *et al*., 2019). India is the major producer of dry chilli with an annual production of 1.2 million tonnes followed by China with around 0.25 million tonnes (FAOSTAT, 2020). Nematodes are devastating parasites of crop plants in agricultural production and certainly contribute significantly to net reduction in yield. Nearly every crop in the world is attacked by root-knot nematodes (RKN), making them the most commercially significant group of plant parasitic nematodes (Sasser and Freckman, 1987). *M. incognita* infection severely damages the root system and cause huge economic losses in pepper (Thies *et al*., 1998).

Managing RKN through host plant resistance is a cost-effective farmer-friendly and eco-friendly approach.. Resistance to phytoparasitic nematodes is commonly associated with hypersensitive reaction (HR), a rapid and localised cell death in the sick plant in response to nematode attack. Reactive oxygen species (ROS) are essential for plant defense mechanism, and resistant plants frequently have higher levels of ROS-detoxifying enzymes like peroxidase (PO) and catalase (CAT) when a pathogen attack is occurring. Plants produce more ROS as a result, and when these ROS build up in plant cells, HR occurs. Hydrogen peroxide plays a crucial role in the initiation of HR in interactions that are incompatible. Antioxidant enzymes including peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), super oxide dismutase (SOD), and catalase (CAT) are among the most important protective enzymes involved in the removal of free radicals and activated oxygen species and associated with disease resistance mechanisms (Chandrawat *et al,.*2020).

Polyphenols are believed to seal wounds or diseased tissue, thereby preventing secondary infection or infection spread. Polyphenols cause the darkening of tissue during lesion development (Vaughn *et al.,* 1988; Mayer and Harel, 1979). Plants' phenol metabolism and hypersensivity response are defense systems against invading harmful organisms such as nematodes. The increase in polyphenol oxidase following nematode entry attributes to pathogen-induced phenol oxidation mechanism (Maraite, 1973).Also, plants produce a number of proteins in response to pathogen attack. Pathogenesis-related (PR) proteins are host-encoded proteins generated in response to pathogen invasion, stress, or elicitor treatment (Van Loon, 1997). Systemic acquired resistance has been connected with the creation of PR proteins (SAR).

Since chilli plants are vulnerable to RKN damage, identifying the resistant lines and understanding their mechanism of action helps in efficient management of RKN. Further identified lines can also be explored as root stocks for cultivation of bell/sweet peppers under protected cultivation. Keeping this in view, the current

research aims to assess the biochemical mechanism of resistance exhibited in advanced resistant lines as a function of activities of antioxidant enzyme and changes in biochemical composition, when infected by nematodes.

## MATERIALS AND METHODS

The studies were carried out at the Division of Crop Protetion, ICAR - Indian Institute of Horticultural Research (IIHR), Bengalurufrom July 2021 to September 2022 in Bengaluru. Two identified resistant advanced breeding lines (ACRIL90, ACRIL70) and susceptible (Arka Mohini, Arka Suphal) lines were studied to elucidate the mechanism of action of resistant lines against *M. incognita.*

### Nematode collection

Egg masses were collected from culture plants of tomato (cv. Arka Rakshak) maintained in Nematology glass house, Division of Crop Protection, ICAR –IIHR, Bengaluru. Second stage juveniles (J2) hatching out from the eggs were harvested every day, and only J2 not less than 5 days old were utilized for inoculation.

### Nematode inoculation

In a completely randomised design with six replications, two resistant lines were sown in polybags in the net house with inoculated and uninoculated treatments. These plants were uprooted 15 days and 30 days after inoculation, and the following chemical compositions were estimated in both resistant and susceptible plants under both control and inoculated conditions: The biochemical changes occurring in resistant lines were analysed by studying the total phenols, lignin content, protein content and defence enzyme activities.

The seeds of the selected resistant advanced breeding lines (ACRIL90, ACRIL70) along with susceptible varieties (Bell pepper; Arka Mohini, chilli; Arka Suphal) were surface sterilized for 5 minutes with 0.1% $HgCl_2$, then carefully rinsed with sterile water and air dried. They were then sowed in Coco peat trays and maintained with regular watering. Four weeks old seedlings were transplanted to black nursery polybags (1 kg capacity) filled with sterilized potting mixture containing red soil, FYM and sand in 1:1:1 proportion. In a completely randomised design with six replications, the lines were arranged with nematode inoculated and uninoculated treatments, For challenge inoculation with nematodes, second stage juveniles of *M. incognita* were inoculated into three 2 cm deep holes in the soil around the stem base @ 1000 J2 per plant. After 15 and 30 days, the plants were pulled out and the roots were rinsed, and the following enzymatic and biochemical composition were measured to evaluate their response to nematodes.

### Enzyme extraction

Frozen root sample (1 g) was grinded in a mortar and pestle with 10 mL of 0.05 M phosphate buffer (pH 7.0) containing 10% (w/v) polyvinylpyrrolidone (PVPP) and 0.1 M EDTA. Homogenates were centrifuged (15000g, 15 min, 4 °C) and supernatants used for enzyme assays.

### Superoxide Dismutase (SOD)

Methionine, nitroblue tetrazolium (NBT), EDTA, $Na_2CO_3$, phosphate buffer, and distilled water comprised the reaction mixture. The enzyme extract was added last, followed by addition of 0.1 ml of riboflavin to initiate the reaction. The intensity of the produced colour was measured at 560nm (Xing *et al.,*2008)**.**

### Polyphenol Oxidase (PPO)

To facilitate enzyme extraction, phosphate buffer was added. The reaction was started by adding 2% of catechol. PPO activity was assayed by measuring the linear increase in absorbance at 410 nm by following the method of Augustin *et al*. (1985).

### Peroxidase (PO)

Enzyme extract was added to chilled guaiacol The reaction was started by adding $H_2O_2$ and the rate of decrease in absorbance at 470 nm was measured at 30 seconds intervals for 3 mins. The unit of enzyme is defined as a decrease in O.D by 1.0 under standard conditions by following Shannon *et al*. (1966) method.

### Determination of total phenol content

Total phenol content was estimated by the method described by Skerget *et al.* (2005). Plant extract was prepared by adding 400 mg of dried plant sample to 60% ethanol. Then, 2 mL of plant extract was added to Folin-Ciocaltaeu reagent and 8 mL of $Na_2CO_3$. Incubation was performed for 2 h, and absorbance was recorded at 765 nm.

### Determination of protein content

About 0.5g of root tissue was taken and thoroughly grinded with 5ml of water. The extract was centrifuged for 10 min at 10000 rpm. Then, 0.1ml of supernatant was taken in clean test tubes and the volume made up to 1ml by adding distilled water. To this was added 5 ml of reagent 4 (Alkaline copper solution), mixed thoroughly and allowed standing at RT for 10 min. The, 0.5 ml Folin-Ciocalteau's reagent (1:1) was added and incubated all the tubes for 30 min at dark. The intensity of the blue colour developed was read by measuring the

absorbance at 750 nm. Results were expressed as mg of bovine serum albumin (BSA) equivalents per 100 g of fresh weight (Lowry *et al.*, 1951).

**Quantification of lignin**

The ash content of all samples was determined by burning the insoluble fraction for 4 hours in a muffle furnace at 550 °C. Lignin from the insoluble fraction was calculated by the difference in the weight of the dry mass and the total ash for each sample. The lignin content was determined by the sum of the insoluble and soluble lignin and was expressed as mg/g21 cell wall following the method of Abdelrahman (2018).

**Results and discussion**

The results on defense enzymes revealed significantly higher acitivity in resistant lines than the susceptible ones, with enhanced expression when inoculated with nematodes than the uninoculated plants.

**Super oxide dismutase (SOD)**

The analysis of nematode resistant and susceptible rootstocks for super oxide dismutase activity revealed that the inoculated resistant lines expressed higher activity with 10.009mg and 16.923mg in ACRIL 70; 9.987 mg and 14.363mg in ACRIL 90 at 15 and 30 days after inoculation, respectively. The uninoculated resistant lines showed lower SOD activity signifying the elevation of SOD activity for defense during the time of infection.

Even in the susceptible varieties, inoculated Arka Mohini and Arka Suphal exhibited greater SOD activity than the un-inoculated plants with 7.908 mg and 8.783 mg, respectively, at 15 DAI. However, at 30 DAI, the SOD activity was recorded to be higher in the susceptible uninoculated plants with 10.245 mg and 11.512 mg than the inoculated plants with 9.08 mg and 9.236 mg values for Arka Mohini and Arka Suphal, respectively (Table 1 and 2). Similar results were also reported by Kashyap *et al.,* (2021), where SOD activity was raised in all treatments 24 hours after pathogen injection. Neena Chawla *et al*. (2013) also reported similar results, indicating that SOD activity increased in the roots and leaves of inoculated and uninoculated plants of resistant and susceptible genotypes.

**Peroxidase**

The resistant (ACRIL 70 and ACRIL 90) lines inoculated with nematodes recorded with higher peroxidase activity values compared to both the uninoculated resistant lines as well as the susceptible check varieties. The results showed that at 15 and 30 DAI, in both the resistant genotypes, enzyme activity was increased than that of susceptible genotypes in both inoculated and uninoculated treatments. While in susceptible genotypes decreased activity was observed when compared with resistant genotypes.

The resistant inoculated lines showed peroxidase activity as high as 9.233µg in ACRIL 70 and 8.905µg in ACRIL 90 after 15 days and still higher as 14.312µg (ACRIL 70 ) and 11.276µg (ACRIL 90) after 30 days of inoculation. Concurrently the uninoculated ACRIL 70 recorded 4.428µg, 8.879µg and ACRIL 90 recorded 4.264µg, 8.106µg at 15 and 30 days after inoculation, respectively. Among the susceptible Arka Mohini and Arka Suphal also, the inoculated varieties recorded higher peroxidase activity than the uninoculated, both at 15 and 30 DAI (Table 1 and 2).

This strengthens the point that the nematode infection elevates the defense mechanism of peroxidase activity that helps to speed up the polymerization process by which phenolic chemicals are transformed into lignin as observed by Gaspar *et al.* (1982). When a pathogen attacks, a plant's primary defense is to begin producing new cell walls. Plants contain many peroxidase isoenzymes that vary in how they react with substrates and how they are constructed. These results are in line with the findings of Pankaj *et al*. (1994) in which the highest compared to DL 482, the susceptible line.

**Poly phenol oxidase (PPO)**

PPO is a part of primary defense mechanism, thus the same has been exhibited in the present study. Table 1 and 2 demonstrates the obtained results for the PPO content. The inoculated resistant lines, ACRIL 70 and ACRIL 90 showed a higher accumulation of PPO i.e. 2.006mg, 1.730mg and 3.213mg, 3.010mg at 15 and 30 DAI, respectively. Meanwhile, the uninoculated resistant ACRIL 70 (1.260mg, 1.026mg) and ACRIL 90 (2.486mg, 2.180mg) had lower PPO content.

On the other hand, significantly lesser PPO content was observed in the susceptible varieties, Arka Mohini (1.310mg,1.023mg) and Arka Suphal (1.416 mg,1.320 mg) both at 15 and 30 DAI leading to the conclusion that susceptible varieties succumbed to the infection and hence, lacked this defense mechanism. The variation can be clearly understood within the inoculated and uninoculated susceptible varieties showing reduction in PPO content in inoculated plants than in the uninoculated ones. These results were positively correlated with the findings of Kosuge (1969) that PPO catalyzes the hydroxylation of mono phenols to diphenols and the oxidation of diphenols to quinones which rapidly polymerize to produce black or brown pigments

**Table 1. Biochemical changes in resistant and susceptible genotypes at 15 DAI of root knot nematodes**

| 15 DAI | Phenols (mg/g FW) | Lignin (Ash %) | Proteins (µg/g FW) | PO (units/µg protein) | SOD (units/mg protein) | PPO (units min-1mg-1 FW) |
|---|---|---|---|---|---|---|
| **Resistant** | | | | | | |
| ACRIL-70 U | 0.619 | 65.3 | 0.551 | 4.428 | 7.377 | 1.26 |
| ACRIL-70 I | 0.716 | 61.1 | 0.575 | 9.233 | 10.009 | 2.006 |
| ACRIL-90 U | 0.581 | 68.2 | 0.513 | 4.264 | 6.87 | 1.026 |
| ACRIL-90 I | 0.692 | 50.7 | 0.557 | 8.905 | 9.987 | 1.73 |
| **Susceptible** | | | | | | |
| Arka Mohini U | 0.308 | 49.7 | 0.326 | 3.314 | 5.868 | 2.23 |
| Arka Mohini I | 0.581 | 31.4 | 0.471 | 6.413 | 7.908 | 1.31 |
| Arka Suphal U | 0.341 | 52.3 | 0.391 | 3.338 | 5.984 | 2.64 |
| Arka Suphal I | 0.524 | 32.23 | 0.489 | 6.604 | 8.783 | 1.416 |
| CD at 5% | 0.009 | 3.551 | 0.0056 | 0.034 | 0.491 | 0.122 |
| SEm | 0.003 | 1.174 | 0.003 | 0.011 | 0.162 | 0.04 |

I= Inoculated U= Un inoculated FW= Fresh Weight; DAI – Days after Inoculation

(polyphenols). PPO activity increases in virus, bacteria, fungi and nematode infected tissues and similar findings was observed by Brueske and Dropkin (1973).

**Phenol content**

The study revealed that the phenol content was higher in resistant lines i.e., ACRIL 70 and ACRIL 90 both at 15 and 30 DAI of nematodes with 0.716mg, 0.920mg and 0.692mg, 0.980mg, respectively. In comparison the susceptible lines under the experiment, Arka Mohini and Arka Suphal recorded only 0.581 mg, 0.618mg and 0.524mg, 0.606mg at 15 and 30 DAI, respectively. The variation in the phenol content within the resistant lines revealed that the inoculated lines showed higher phenol accumulation, than the uninoculated lines both at 15 and 30 days intervals (Table 1 and 2). The trend of higher phenol accumulation in inoculated plants was followed in the susceptible lines as well, indicating increase in phenol content as a mechanism favouring resistance. Similar kind of results were observed by Pandey *et al.* (2016) that increased amount of phenol content was observed in resistance genotypes than susceptible genotypes during infection by RKN in greengram.

**Protein content**

Higher protein content was observed in nematode inoculated resistant lines than susceptible lines after 15 and 30 DAI. In inoculated resistant lines with 0.575 mg, 0.557 mg (15 DAI) and 0.684 mg, 0.667 mg (30 DAI) as against the uninoculated resistant lines with 0.551 mg, 0.513 mg (15 DAI) and 0.487 mg, 0.472 mg (30 DAI) (Table 1 and 2). The results were on par with the findings of Gopinath *et al.* (2002) in which the moderately resistant tomato cultivars, Vivek and Radha recorded maximum concentration of proteins, which confer resistance to RKN infection whereas in susceptible cultivar Pusa Ruby the protein concentration is less as compared to the resistant cultivars.

**Lignin content**

Lignin concentration was relatively higher in resistant lines compared to the susceptible lines however uninoculated lines revealed more lignin concentration than the inoculated ones. . This consequently establishes the fact that the nematodes on infection damage the cell wall and ultimately leads to reduction in the lignin content. The damaged cells also show less pronounced translation subsequently producing lower levels of protein. The resistant lines ACRIL 70 and ACRIL 90 showed lignin content with 61.1% ash and 50.7% ash in the inoculated lines, while the uninoculated lines had 65.3 % ash, 68.2 % ash of lignin at 15 DAI. At 30 DAI lignin content of 60.2 % ash, 54.0 % ash in inoculated and 65.2 % ash, 63.8 % ash in uninoculated lines, respectively were recorded. These results were coincided with the results obtained by Tian *et al.* (2022) revealing that *C. chinense* showed higher lignin content in resistant genotype than susceptible after the *M. enterolobii* inoculation.

## CONCLUSION

Understanding biochemical basis of resistance in roots against root knot nematodes will facilitate in identification of candidate biochemical markers for indirect selection of genotypes with resistance. Based on the results we can conclude that the defense enzymes (SOD, PPO and PO) and biochemical constituents

**Table 2. Biochemical changes in resistant and susceptible genotypes at 30 DAI of root knot nematodes**

| 30 DAI | Phenols (mg/g FW | Lignin (Ash %) | Protiens (µg/g FW) | PO (units/µg protein) | SOD (units/mg protein) | PPO (units min-1mg-1 FW) |
|---|---|---|---|---|---|---|
| **Resistant** | | | | | | |
| ACRIL-70 U | 0.780 | 65.2 | 0.487 | 8.879 | 14.713 | 2.486 |
| ACRIL-70 I | 0.920 | 60.2 | 0.684 | 14.312 | 16.923 | 3.213 |
| ACRIL-90 U | 0.683 | 63.8 | 0.472 | 8.106 | 13.42 | 2.18 |
| ACRIL-90 I | 0.980 | 54 | 0.667 | 11.276 | 14.363 | 3.01 |
| **Susceptible** | | | | | | |
| ArkaMohini U | 0.423 | 56.3 | 0.221 | 5.577 | 10.245 | 2.003 |
| Arka Mohini I | 0.618 | 32.6 | 0.491 | 9.843 | 9.087 | 1.023 |
| Arka Suphal U | 0.440 | 52.7 | 0.277 | 5.979 | 11.512 | 2.106 |
| Arka Suphal I | 0.606 | 27.3 | 0.494 | 9.986 | 9.236 | 1.32 |
| CD at 5% | 0.008 | 3.299 | 0.0042 | 0.072 | 0.424 | 0.07 |
| SEm | 0.003 | 1.091 | 0.004 | 0.024 | 0.14 | 0.023 |

I= Inoculated U= Uninoculated FW= Fresh Weight; DAI – Days after Inoculation

(Phenols, lignin and protein) were increased in the roots of resistant lines compared to that of susceptible genotypes after RKN inoculation. Advanced breeding lines ACRIL 70 and ACRIL 90 showed increased resistance to RKN both phenotypically and biochemically it can be further utilized for the breeding to incorporate the resistance into the elite genotypes. These genotypes can serve as potential root stock for chilli/capsicum to mitigate the root knot nematode problem in the infected soils.

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