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# INDUCTION OF PHENYLALANINE AMMONIA LYASE IN THE SEEDLINGS OF CUCUMBER AND CHILLI BY THE ELICITORS IS SPECIES- AND TISSUE- SPECIFIC

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Cucumber and chilli face huge loss of yield due to various etiologies of diseases. Hence, fungicides and pesticides are used to control diseases, which pose environmental threats. Induction of systemic acquired resistance (SAR) of plants by chemicals is an environment-friendly alternative of protecting plants against invading pathogens. SAR is characterized by synthesis of antifungal proteins, production of phytoalexins and accumulation of phenolic compounds. These phenolics compounds play a pivotal role in increasing the resistance mechanisms of plants. Thus, present investigation was planned to evaluate the activity of Phenylalanine ammonia layase (PAL) induced in response to abiotic and biotic elicitors in cucumber and chilli. We found that all the elicitors viz., copper sulphate, mercuric chloride, salicylic acid, UV light and fungal cell wall elicitors prepared from the fungal cultures of *Fusarium oxysporum* Schlecht and *Colletotrichum gleosporioides* Penz used, induced PAL activity to a significantly high-level. The response of seedlings towards elicitation was initially low, which gradually increased after 30h of elicitation and the peak activity of PAL was recorded between 45 to 60h after elicitation. The time needed for PAL activity to reach its peak depended on the species, elicitor and seedling part.

**Keywords :** *Capsicum, Cucumis,* elicitors, plant defence, SAR

Cucumber (*Cucumis sativus* L.) and chilli (*Capsicum annuum* L.) are two economically important crops of India. However, both these crops face huge loss of yield due to various pathogens. In India, a number of major and minor cucurbits are cultivated, which share about 5.6% of the total vegetable production. Over 200 known cucurbit diseases of diverse etiologies are known , which may reduce the yield by 50-70%. Chilli, another important crop is vulnerable to diseases. Anthracnose disease caused by *Colletotrichum* species, stem rot caused by *Rhizoctonia* solani, bacterial wilt caused by *Pseudomonas solanacearum* and mosaic disease caused by chili veinal mottle virus (CVMV) or Cucumber mosic virus (CMV) are the major constrains to chili production .

To control yield loss due to disease attack, several pesticides, fungicides and insecticides are being used. However, it not considered to be a long-term solution due to concerns of expenses, its residues, toxicity to non-target organisms and other health and environmental hazards. Hence, increasing resistance seems to be the best option. Conventional breeding is a good alternative but it takes much time and labor to develop a resistant variety. Therefore, recent efforts are focused on developing ecofriendly, safe, long-lasting and effective management of plant diseases by exploiting the known elicitors. Elicitors are highly specific molecules produced by plant pathogen at low concentrations which are capable of inducing systemic resistance in plants. Use of elicitors for disease management requires standardization of chemicals, timing and concentration required for elicitation of SAR in plants.

Phenylalanine ammonia lyase (PAL) is the key enzyme in phenyl propanoid metabolism and plays a significant role in the regulation of biosynthesis of phenols in plants. PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid. The products of this pathway have diverse function in plants. Notably in defense, the functions are cell wall strengthening and repair (e.g. lignin, suberin and phenolics) and antimicrobial activity (e.g. furanocoumarins etc.). Several studies indicate that the activation of PAL and subsequent increase in phenolic contents in plants is a general response associated with disease resistance. Apart from pathogens, PAL activity can be induced by various abiotic and biotic stresses also.

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Hence, the present study was planned, to test the efficacy of abiotic and biotic elicitors in induction of PAL and thus contributing to development of SAR in cucumber and chilli.

## MATERIALS AND METHODS

### Procurement of seeds

The seeds of cucumber (var. Mohini 186-51585) and chilli (var. Pusa jwala 172-25624) were provided on gratis by Ankur Seeds Pvt. Ltd, Nagpur.

### Raising the seedlings

The seeds were surface sterilized by washing them sequentially with 0.1% $HgCl_2$ and 70% alcohol for one minute each. Then the seeds were thoroughly washed with sterile distilled water thrice. About 10-15 seeds were inoculated in sterilized test tubes (3.2 × 20cm) containing 1/2 strength agarized basal MS medium. In case of chilli the seeds were pre-soaked in 500ppm gibberellic acid solution prior to inoculation over 1/6th agarized basal MS media. The tubes were incubated in dark at constant temperature at 25 ± 2°C until used.

### Elicitor preparation

Chemical elicitors used in the present investigation were aqueous solution of copper sulphate (0.5mM), mercuric chloride (0.5mM) and salicylic acid (0.25mM, 0.5mM and 0.75mM). Besides this a physical elicitor, UV light, was also used. Biotic elicitor was prepared from the cell wall of fungal pathogen infecting the plant. For this the fungal cultures of *Fusarium oxysporum* Schlecht (NFCCI-745) and *Colletotrichum gleosporioides* Penz (NFCCI-1734) infecting cucumber and chilli, respectively were procured from Agharkar Research Institute, Pune (India). The fungal strains were maintained over potato dextrose agar (PDA). Fungal cell wall elicitor was prepared using three-week-old cultures grown in liquid medium essentially as described by (Badere *et al.* 2007, Kadoo and Badere 2014). The total carbohydrate content of the cell wall elicitor in terms of glucose equivalent was determined by Anthrone reagent method. The elicitor containing 50µg glucose equivalent/ml was used to study the induction of PAL.

### Elicitation of seedlings

The seedlings were elicited on 12th day in case of cucumber, whereas on 21st day in case of chilli before the appearance of true leaves and when the cotyledonary leaves were still turgid. Dark grown seedlings were elicited by aseptically spraying them with abiotic and biotic elicitors. Seedlings were elicited with UV light by exposing them to it for 10 minutes at the distance of 30 cm in a laminar airflow bench. After elicitation the seedlings were frozen in liquid nitrogen at an interval of 5h up to 75h.

### Preparation of crude enzyme extract

The crude enzyme extract was prepared by homogenizing 1g frozen tissue in 1ml of ice-cold extraction buffer (50mM Tris-HCl pH 8.5) in pre-chilled mortar and pestle kept in an ice bucket. Polyvinyl pyrrolidone (PVP) was added at the time of extraction to adsorb phenolics, particularly cinnamic acid, which interferes with spectrophotometric readings. The extract was centrifuged at 10,000 rpm for 10min and the clear supernatant was used as the enzyme source for the assay.

### Enzyme assay

The activity of Phenylalanine ammonia lyase (EC 4.3.1.24) was assayed according the procedure given by. The assay mixture containing 1mM L-Phenylalanine and 300µl of enzyme extract was incubated for 60min at 30ºC. The reaction was arrested by adding 2N HCl. Later, 1.5ml of toluene was added to it and vortexed for 30s, centrifuged at 1,000 rpm for 5min and the toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against toluene blank. To prepare standard curve, different concentrations of cinnamic acid ranging from 10-100µg were prepared and their absorbance was read at 290nm. The total protein content of the crude enzyme extract was estimated by the protein dye binding method given by Bradford. The enzyme activity was then calculated using the total quantity trans-cinnamic acid released during the reaction and the quantity of protein

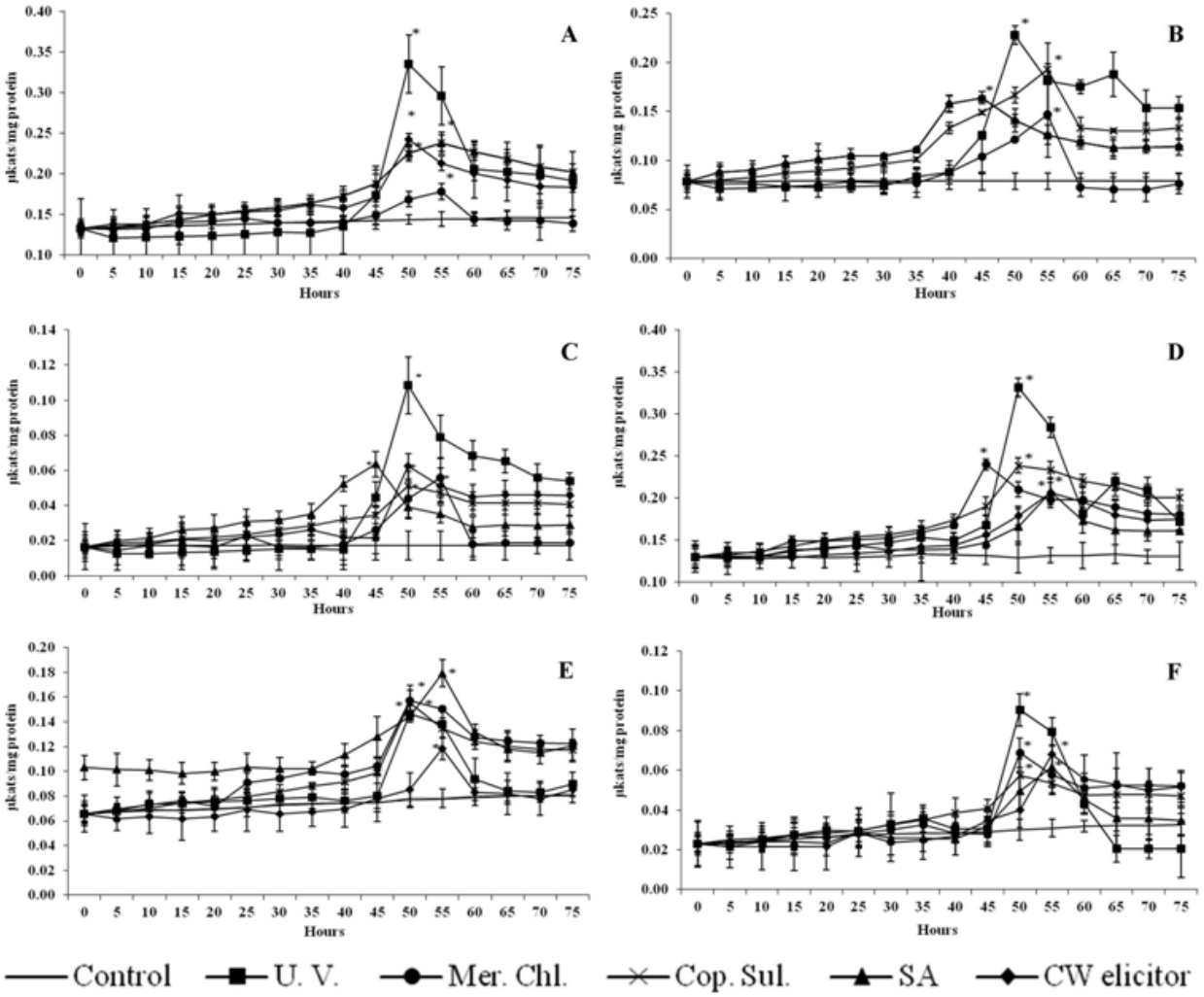


**Figure 1 :** Induction of Phenylalanine ammonia lyase activity in the seedling of cucumber and chilli.
**Cucumber : A**–Cotyledonary leaf, **B**–Hypoeotyl, **C**–Root, **Chilli : D**–Cotyledonary leaf, **E**–Hypocotyl, **F**–Root
*Significant at 5% level of confidence by Student's t-test; Mer. Chl. at 5% level of confidence by Student's t-test; Mer. Chl.–Mercuric chloride, Cop. Sul–Copper sulphate, SA–Salicylic acid.

present in the crude enzyme extract.

**Statistical analysis**

Computation of mean, standard error, Student's t-value, ANOVA, and Duncan's multiple range test (DMRT) was performed using MS-Excel and XL-STAT.

**RESULTS**

Different authors have used different concentration of salicylic acid to elicit the defence response. Therefore, initially we used three different concentrations of salicylic acid viz. 0.25, 0.50 and 0.75mM for elicitation. We found that at lower two concentrations salicylic acid did not elicit the enzyme activity (data not shown) but at 0.75mM it did. Thus, we carried out the final experiments using this concentration of salicylic acid.

The activity of PAL was induced gradually after the elicitation of seedling. Its peak activity was recorded between 45 to 60h after elicitation. The time needed for PAL activity to reach its peak depended on the species, elicitor and seedling part. UV light proved to be the best elicitor in triggering the activity of PAL in both plants. It was followed by salicylic acid and cell wall elicitor (CWE) which induced PAL activity to an appreciable level. However, mercuric chloride and copper sulphate were weakest in inducing PAL (Fig. 1).

First of all, to have an assessment of an

**Table 1.** Peak Phenylalanine ammonia lyase activity induced by various elicitors in cucumber seedlings.

| Explant | Control | U.V. | Mercuric chloride | Copper sulphate | Salicylic acid | Cell wall elicitor |
|---|---|---|---|---|---|---|
| **Cotyledonary leaf** | $0.13^{h\,6} \pm 0.004$ | $0.38^{a\,1\,i} \pm 0.004$ | $0.23^{f\,5\,i} \pm 0.004$ | $0.25^{d\,4\,i} \pm 0.004$ | $0.32^{b\,2\,i} \pm 0.004$ | $0.26^{c\,3\,i} \pm 0.004$ |
| **Hypocotyl** | $0.08^{k\,6} \pm 0.004$ | $0.24^{e\,1\,ii} \pm 0.02$ | $0.19^{g\,2\,ii} \pm 0.035$ | $0.13^{h\,4\,ii} \pm 0.003$ | $0.19^{g\,5\,ii} \pm 0.003$ | $0.19^{g3\,ii} \pm 0.003$ |
| **Root** | $0.02^{m\,6} \pm 0.002$ | $0.11^{i\,1\,iii} \pm 0.004$ | $0.11^{i\,2\,iii} \pm 0.004$ | $0.06^{l\,5\,iii} \pm 0.002$ | $0.07^{l\,4\,iii} \pm 0.002$ | $0.10^{j\,3\,iii} \pm 0.004$ |

Mean(s) with same letter in the table are not significantly different at 5% using DMRT.*; Mean(s) with same Arabic numeral in same row are not significantly different at 5% using DMRT. *; Mean(s) with same Roman numeral in same column are not significantly different at 5% using DMRT. *

**Table 2.** Peak Phenylalanine ammonia lyase activity induced by various elicitors in chilli seedlings.

| Explant | Control | U.V. | Mercuric chloride | Copper sulphate | Salicylic acid | Cell wall elicitor |
|---|---|---|---|---|---|---|
| **Cotyledonary leaf** | $0.13^{j\,6} \pm 0.009$ | $0.37^{a\,1\,i} \pm 0.006$ | $0.23^{d\,2\,i} \pm 0.005$ | $0.25^{b\,3\,i} \pm 0.005$ | $0.24^{e\,4\,i} \pm 0.008$ | $0.21^{e\,5\,i} \pm 0.009$ |
| **Hypocotyl** | $0.08^{0\,6} \pm 0.006$ | $0.19^{f\,1\,ii} \pm 0.003$ | $0.19^{f\,2\,ii} \pm 0.006$ | $0.16^{i\,5\,ii} \pm 0.005$ | $0.18^{g\,3\,ii} \pm 0.006$ | $0.17^{h\,4\,ii} \pm 0.005$ |
| **Root** | $0.03^{q\,6} \pm 0.003$ | $0.12^{k\,1\,iii} \pm 0.004$ | $0.1^{m3\,iii} \pm 0.004$ | $0.06^{p\,5\,iii} \pm 0.004$ | $0.09^{n\,4\,iii} \pm 0.005$ | $0.1^{l\,2\,iii} \pm 0.008$ |

Mean(s) with same letter in the table are not significantly different at 5% using DMRT.*; Mean(s) with same Arabic numeral in same row are not significantly different at 5% using DMRT. *; Mean(s) with same Roman numeral in same column are not significantly different at 5% using DMRT. *

overall response of seedling parts towards elicitation, two-way ANOVA was performed using plant-part and elicitor as the factors followed by DMRT. In both the crop species the weakest response was displayed by root in almost all the cases. Similarly, with certain exceptions, the strongest response was given by cotyledonary leaf in both the crops. The response of seedling parts was uniform in terms of induction of PAL. The strongest response was posed by cotyledonary leaf towards all elicitors. It was followed by hypocotyl and root in both the crops.

Maximum PAL activity was induced in cucumber by UV light followed by salicylic acid in cotyledonary leaf (Table 1), while in chilli UV light induced maximum PAL activity in cotyledonary leaf followed by copper sulphate (Table 2). The maximum activity was evoked by UV light in all the parts of the seedlings of both the plants. Unlike, the most effective elicitor, the weakest elicitor was found to be different in each case. The observation worth mentioning here is that mercuric chloride was weakest only in cotyledonary leaf of cucumber.

In the next step we tested the response of each part of seedling towards different elicitors. Both the plants responded in the similar manner in inducing PAL activity in response to the elicitor treatment. In cotyledonary leaf the maximum response was evoked by UV light with the activity of 0.38µKats/mg protein, while the minimum response was with mercuric chloride the activity being 0.23µKats/mg protein (Table 1). Although, UV light induced about same level of PAL activity i.e. 0.37µKats/mg protein in chilli, as

in cucumber, but the weakest response was induced by CWE with activity of 0.21µKats/mg protein (Table 2). In hypocotyl of cucumber maximum, PAL activity was triggered by UV light (0.24µKats/mg protein). UV light was followed by mercuric chloride, salicylic acid and CWE, which induced PAL activity of 0.19µKats/mg protein. However, copper sulphate was found to be weaker, which induced the activity of 0.13µ Kats/mg protein (Table 1). The response of chilli did not vary much towards the elicitors used. The maximum response of chilli was 0.19µ Kats/mg protein (UV light and mercuric chloride) and the minimum was 0.16µ Kats/mg protein (copper sulphate) (Table 2).

The PAL activity was induced in roots of both the crops more or less equally. Moreover, the range of activity induced by the elicitors was also narrow. The most efficient elicitors in cucumber were UV light and mercuric chloride with the PAL activity of 0.11µKats/mg protein. The weakest elicitor in cucumber was found to be copper sulphate, which induced PAL activity of 0.06µKats/mg protein (Table 1). Similarly, the strong and weak response in chilli was induced by UV light (0.12µ Kats/mg protein) and copper sulphate (0.06µ Kats/mg protein), respectively (Table 2).

## DISCUSSION

In the present investigation various abiotic and biotic elicitors were tested for induction of PAL activity in the seedling of cucumber and chilli. The peak activity of PAL was recorded between 45 to 60h after elicitation. The time needed for PAL activity to reach its peak depended on the species, elicitor and seedling part. Amongst elicitors used, UV light proved to be the best in eliciting PAL activity in both the plants. It has been demonstrated by in cucumber that exposure of seedlings to UV-B radiation caused 78% increase in activity of PAL. Similar reports were made by while working on Phaseolus trilobus seedlings treated with UV light. stated that UV-C light treatment induces resistance to infection in several crops including some harvested commodities. Next to UV light, salicylic acid and CWE also induced PAL activity to an appreciable level. The role of salicylic acid in induction of plant defense response is universally known. It is involved in signal transduction systems to induce defense enzymes. As an elicitor, salicylic acid regulates the PAL enzyme activity. The results of present investigation are in agreement with who demonstrated the induction of PAL activity by 0.8mM salicylic acid in two different cultivars of chickpea.

Fungal CWE was effective in inducing PAL activity in present investigation. These results are in confirmation with the earlier reports made by, where the CWE derived from *Cercosporidium personatum* and *Phoma eupyrena* induced PAL activity in groundnut cultivars. Similarly, also reported the use of CWE and spore suspension of *Cercospora canescens* to induce PAL activity in mungbean. Apart from CWE, other biotic elicitors have also been reported to trigger the PAL activity in plants. reported induction of PAL in *Solanum tuberosum* by a 10kDa protein named 'infestin' produced by *Phytopthora infestens*. Moreover, they also reported purified infestin to be superior in inducing PAL activity than its crude preparation. have demonstrated the induction of PAL activity by spore suspension of *Fusarium oxysporum* f. sp. lycopersici.

In the present investigation, different seedling parts expressed PAL differentially upon elicitation with an elicitor. Additionally, PAL expression was found to vary in the two plants. Cotyledonary leaf exhibited highest PAL activity as compared to hypocotyl and root. The tissue-specific expression of PAL in the present study is in accordance with the findings of Kale and Choudhary (2001) in groundnut. They found the response of hypocotyl to be maximum in resistant cultivar Girnar-1. The tissue-specific expression of PAL genes induced in response to an elicitor, indicates that the cell surface receptors are differentially distributed over the seedling parts. As a result, one tissue is able to recognize one class of elicitor molecule more efficiently than the other. In several other

plants, the plant defense genes have also been shown to be differentially expressed in different tissues. showed tissue- and cell-specific activity of PAL in transgenic tobacco and potato. Their analysis showed that the same promoter region was able to confer developmental specificity and wound inducibility to the gene.

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