

## Enhancement of phenylalanine ammonia-lyase activity in cucumber and chilli seedlings by aqueous extract of *Cleistanthus collinus*

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The aqueous extract of the aerial parts of commonly available plants in Nagpur (India) was screened for their ability to induce the activity of phenylalanine ammonia-lyase (PAL) in cucumber and chilli. *C. collinus* induced PAL activity in both crop species effectively after 55 hours of elicitation. Later, the *C. collinus* extract was fractionated over a silica gel column. The fraction collected with 90% methanol also induced the activity of PAL in the seedlings. However, the fractions eluted with 45 and 0% aqueous methanol failed to induce PAL activity. These findings indicate the efficacy of the extract to trigger active defence responses in cucumber and chilli seedlings. Therefore, *C. collinus* can be useful in developing an organic formulation for crop protection.

**Keywords:** *Capsicum annum*, Crop protection, *Cucumis sativus*, Phytoalexin, SAR

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### Introduction

The pollution caused by synthetic fungicides and the threat posed by such chemicals to the biota of the ecosystem has started a search for a 'green' alternative to these compounds. Among such alternatives, researchers have extensively investigated plant extracts. The plant extracts act via two modes, viz. direct antifungal action, which inactivates the invading pathogen<sup>1</sup> and stimulation of the plant's intrinsic defence mechanism, which counteracts the pathogen by the active defence responses of the plant<sup>2</sup>. However, workers prefer the latter mode of action because it confers a long-lasting resistance with a wide spectrum of action. Hanaa *et al.* reported the induction of defence-related antioxidant enzymes such as peroxidase, catalase and superoxide dismutase by neem and willow extract in 4-week-old tomato seedlings<sup>3</sup>. The induced resistance effectively controlled the wilt caused by *Fusarium oxysporum* in the plant.

Similarly, the aqueous and ethanolic extracts of *Datura metel* are reported to confer systemic resistance to rice seedlings<sup>4</sup>. The treatment enhanced the activity of the defence-related enzymes and

maintained their high level throughout the study period. Moreover, inoculation of the primed seedlings with *Rhizoctonia solani* enhanced the levels of activity of these enzymes. Recently, Naz *et al.* studied the effect of plant extract and its combination with chemical fungicide (mefenoxam) in wheat against leaf spot blotch pathogen *Bipolaris sorokiniana*<sup>5</sup>. The aqueous and methanolic extract (1.2% w/v) of *Jacaranda mimosifolia* inhibited the growth of the pathogen. Further, the formulation of a 0.6% extract of *J. mimosifolia* reduced the requirement of mefenoxam to 0.1% to act against the pathogen and increased crop yield.

Cucumber and chilli are the two important vegetable crops cultivated worldwide. These crops are vulnerable to several diseases<sup>6,7</sup>. Moreover, being a perishable commodity, the commercially important fruits of these crops are prone to post-harvest losses. The literature survey revealed that studies addressing disease management in cucumber and chilli employing the 'green' alternative are fewer, especially in cucumber. Therefore, we decided to investigate the effect of plant extract in these crops with respect to the induction of defence responses.

We initiated the screening of plant extracts capable of eliciting the defence responses in the seedlings of both crops. For this, the locally available plants viz.,

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*Azadirachta indica*, *Calotropis procera*, *Cleistanthus collinus*, *Dalbergia sissoo*, *Datura innoxia* and *Lantana camara* were selected due to their ease of access to the farmers. We have earlier reported the induction of hydrolases<sup>8</sup> and antioxidant enzymes<sup>9</sup> by the extract of these plants. These defence enzymes confer systemic acquired resistance (SAR) to plants and relieve them from oxidative stress. The magnitude of response towards treating plant extract in the seedlings varied with the seedling part, plant source, concentration of extract and the enzyme being assayed. In each case, the extract of *C. collinus* was most effective and induced qualitative as well as quantitative changes in the enzyme activity.

In the present communication, we report the induction of phenylalanine ammonia-lyase (PAL) activity by extracting these plants in the seedlings of cucumber and chilli. Thus, the present study will aid in developing an eco-friendly method of disease management in cucumber and chilli using local resources. Moreover, this method can be cost-effective and affordable too.

## Materials and Methods

### Plant collection and identification

The aerial parts of the plants viz., *A. indica*, *C. procera*, *C. collinus*, *D. sissoo*, *D. innoxia* and *L. camara* were collected between February and May from the urban and rural areas of Nagpur district, India (20°35' - 21°44' N and 78°15' - 79°40' E). The plants were identified in the Department of Botany, Hislop College, Nagpur, with the help of floras. The collected parts of the plants were used to prepare the extract. For this, the material was initially washed under running tap water. Later, it was shade-dried and pulverised in a grinder. Subsequently, the powder was stored in the zip-lock bags under cool and dry conditions.

### Elicitation of the seedlings

The variety Mohini 186-51585 of *Cucumis sativus* and variety Pusa Jwala 172-25624 of *Capsicum annuum* were received as gifts from Ankur Seeds Pvt. Ltd., Nagpur. The seedlings of both crops were raised and elicited with the aqueous extract of the above plants, essentially as described earlier by Kadoo and Badere<sup>8</sup>, under sterile and etiolated conditions. The seedlings treated with plant extract and distilled water (control) were fixed in liquid nitrogen 0, 50, 55, and 60 hours after elicitation for the PAL assay. In the later part of the study, the extract of *C. collinus* was

fractionated using a silica gel column. These fractions were also used to elicit the seedlings of both crops. These elicited seedlings were also fixed in liquid nitrogen at 0 and 55 hours after elicitation.

### Fractionation of the extract of *C. collinus*

The fractionation of the extract of *C. collinus* was carried out as described by Kadoo and Badere<sup>8</sup>. The extract was sequentially eluted with 90% methanol, 45% methanol and distilled water over the silica gel column. Each fraction was then dried and reconstituted in distilled water in proportion to maintain the original composition of 5 g/100 mL of distilled water.

### PAL assay

The activity of PAL was assayed according to Whetten and Sederoff<sup>10</sup>. The assay mixture comprised 1 μmole of L-phenylalanine and 300 μL of crude enzyme extract. The crude enzyme extract was prepared by homogenising 1 g of seedling tissue in a pre-cooled mortar and pestle in the presence of 1 mL of chilled extraction buffer. The extraction buffer comprised 50 mM Tris-HCl (pH of 8.5) and 14 mM β-mercaptoethanol. About 100 mg of polyvinyl pyrrolidone was added while the tissue was homogenised. The homogenate was subsequently centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant, thus obtained, was used to assay the PAL activity.

The enzyme was allowed to act on the substrate, i.e. L-phenylalanine, at 30°C. The enzyme activity was terminated after 60 min by adding 2N HCl. Subsequently, the *t*-cinnamic acid in the assay mixture was extracted by adding 1.5 mL of toluene. This was then vortexed for 30 seconds and centrifuged at 10,000 rpm for 5 min at 4°C. The toluene layer containing *t*-cinnamic acid was read at 290 nm. The quantity of *t*-cinnamic released in the assay mixture was determined from the calibration curve of cinnamic acid. The protein content of the crude extract was determined by Bradford's protein-dye binding method<sup>11</sup>, and the activity of PAL in the crude enzyme extract was calculated by the following formula:

$$PAL \text{ activity } (\mu\text{kats/mg protein}) = \frac{10 \times c}{5333.76 \times p}$$

where 'c' is the mg of cinnamic acid released during the assay and 'p' is mg protein in the crude enzyme extract.

### Statistical analysis

The statistical computations were done using MS Excel and XL-STAT.

### Results and Discussion

The PAL activity in the seedlings of cucumber and chilli was determined at 0, 50, 55, and 60 hours after elicitation with the plant extracts. The time duration to assay the enzyme activity after elicitation was selected based on our earlier study, where we found that the PAL activity reached its peak around 55 hours after elicitation with abiotic and biotic elicitors<sup>12</sup>. In the present investigation, we elicited the seedlings of cucumber and chilli with 0, 2, 6, 10, 16, and 20% plant extract. The elicitation effect varied with the extract concentration, plant source and crop species. Of all the concentrations tested, 20% extract induced PAL activity in almost all cases. The other concentrations were ineffective (data not shown). The enzyme activity induced by 20% of the extract reached its peak 55 hours after elicitation and later decreased (Fig. 1).

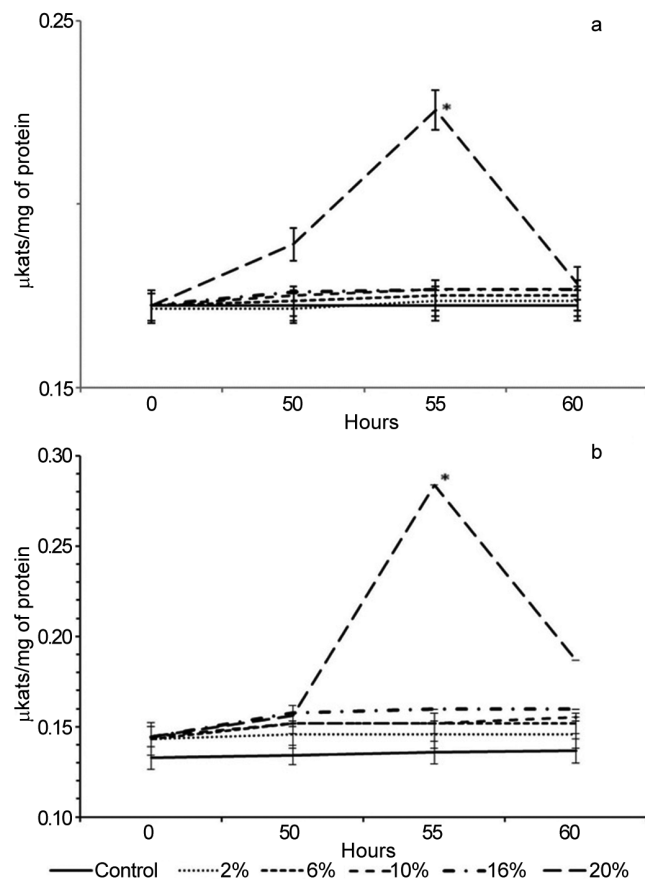


Fig. 1 — Induction of PAL activity in the cotyledonary leaf. a) In cucumber by the aqueous extract of *A. indica*; and b) In chilli by the aqueous extract of *C. collinus*. \*Significant at 5% level of confidence according to Student's t-test.

The products of the phenylpropanoid pathway, of which PAL is a key enzyme, are involved in plant defence. The phenylpropanoids strengthen and repair the cell wall and also possess antimicrobial activity. In addition, the activity of PAL is also implicated in the development of SAR in plants. The signal molecule involved in SAR – salicylic acid – is produced from *t*-cinnamic acid, which is the product of PAL activity<sup>13</sup>. Activation of PAL is associated with disease resistance in plants<sup>14</sup>. Chhabra *et al.* found induction of PAL activity, in addition to other defence enzymes, in rice after the treatment with the extracts of *Azadirachta indica*, *Melia azedarach* and *Toona ciliata*<sup>15</sup>. Similarly, Chaurasiya *et al.* have also reported the induction of PAL activity in the fruits of chilli after the treatment of the extract of *Alternanthera sessilis*, which reduced the deleterious effects of the infection of *Colletotrichum capsici*<sup>16</sup>. Anand and Bhaskaran also found induction of PAL activity in chilli fruits upon the treatment with the extract of *Abrus precatorius*<sup>17</sup>.

Among the plant extracts, the extract of *A. indica* and *C. collinus* induced significant PAL activity in the cotyledonary leaf, hypocotyl and root of both crops. The extract of *C. procera*, *D. inoxia* and *L. camara* was more effective in inducing PAL activity in the seedlings of cucumber compared to chilli. In contrast, the extract of *D. sissoo* effectively induced PAL activity in the cotyledonary leaf of chilli compared to hypocotyl and root (data not shown). Kale and Choudhary also reported tissue-specific induction of PAL activity in groundnut seedlings<sup>18</sup>. They attributed this observation to the tissue-specific distribution of the receptors in the seedling. As a result, certain tissues recognised the elicitor molecule and responded towards it more efficiently compared to others.

Since 20% extract was mostly effective in inducing the PAL activity, we compared the activity induced by 20% extract of all the plants in all three tissues, viz., cotyledonary leaf, hypocotyl and root of cucumber and chilli. Overall, the extract of *C. collinus* was most influential, among all, to induce PAL activity in the seedlings of both crops. The next most effective extract was *C. procera*, followed by *A. indica* (Table 1). This might be because of the chemicals in these plants, which effectively induce the expression of PAL. This assumption is supported by the finding that in the present investigation, only fraction 1 of the *C. collinus* extract eluted with 90% methanol induced the PAL activity in cucumber and chilli (Fig. 2).

Table 1 —The influence of plant extract on the induction of maximum activity of PAL in the seedlings of cucumber and chilli

Plant species	Cucumber			Chilli		
	Cotyledonary leaf	Hypocotyl	Root	Cotyledonary leaf	Hypocotyl	Root
<i>A. indica</i>	0.23	0.11	0.30	0.25	0.12	0.03
<i>C. procera</i>	0.25	0.14	0.05	0.25	0.15	0.06
<i>C. collinus</i>	0.35	0.20	0.12	0.28	0.12	0.04
<i>D. sissoo</i>	0.15	0.09	0.07	0.19	0.10	0.06
<i>D. inoxia</i>	0.15	0.08	0.02	0.14	0.07	0.03
<i>L. camara</i>	0.23	0.15	0.08	0.14	0.01	0.05

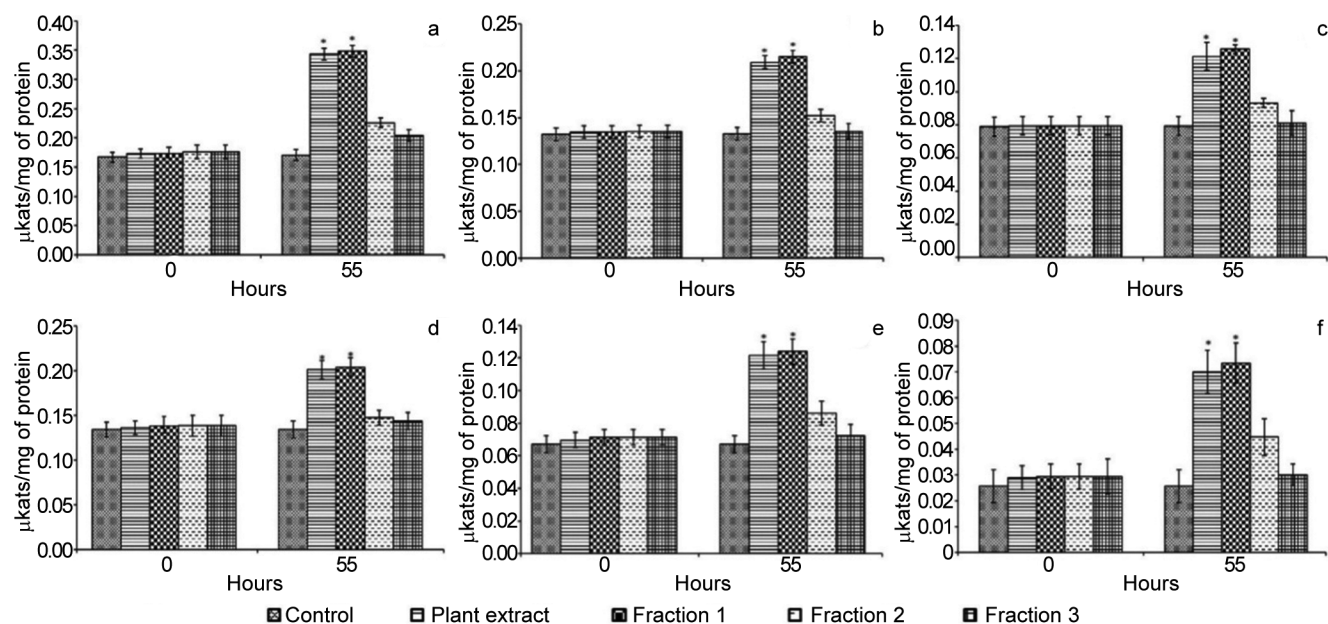
Enzyme activity:  $\mu\text{kats/mg protein}$ 

Fig. 2 — Induction of PAL activity in the cotyledonary seedlings by the extract of *C. collinus* and its fractions eluted over silica gel 60-120 column. Cucumber- a) Cotyledonary leaf; b) Hypocotyl; and c) Root. Chilli- d) Cotyledonary leaf; e) Hypocotyl; and f) Root. \*Significant at 5% level of confidence according to Student's t-test.

Mohamed and El-Hadidy also found only the fractions of *Verbascum eremobium* eluted with butanol and ether to induce the chitinase and peroxidase activity and accumulate phenolics in cucumber<sup>19</sup>. The chemical composition of the fraction eluting out from the column depends on the eluent. Therefore, there is a difference between the bioactivity of each fraction of the extract. The fraction 1 of *C. collinus* was unique in two ways. First, it was richer in phenols and flavonoids, and second, it was diverse in terms of the derivatives of terpenoids, lignans, phenylpropanoids, anthraquinones and saponins<sup>8</sup>. Phenolics are reported to activate the plant's defence mechanism<sup>20</sup>. Similarly, flavonoids<sup>19</sup> and coumarins<sup>21</sup> are proven to activate the defence genes in cucumber and pumpkin, respectively.

Therefore, the effectiveness of fraction 1 in inducing PAL activity in the cucumber and chilli seedlings in the present investigation might be because of the presence of phenolic compounds.

### Conclusion

The whole extract of *C. collinus* and its fraction 1 induced PAL activity in the present investigation. Thus, this indicates the efficacy of *C. collinus* to stimulate phenylpropanoid metabolism in the seedlings, which is involved in the biosynthesis of phytoalexin and salicylic acid. While phytoalexin has the local action, the salicylic acid acts as a signal molecule to develop SAR in plants. Therefore, it is quite possible that a systematic evaluation of the extract of *C. collinus* would lead to the development of a novel organic composition for crop protection.

### Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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