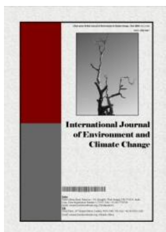

International Journal of Environment and Climate Change

12(10): 1052-1059, 2022; Article no.IJECC.88296
ISSN: 2581-8627
(Past name: British Journal of Environment & Climate Change, Past ISSN: 2231–4784)

Changes in Activity of Phenylalanine Ammonia Lyase (PAL), Phenols and β 1, 3 Glucanase in Crossandra Plants affected by *Fusarium incarnatum* (Desm.) Sacc and treated with Bioagents, Organic Amendments, Silver Nanoparticles and Fungicide in Pot Culture

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJECC/2022/v12i1030898

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/88296

Original Research Article

Received 08 April 2022
Accepted 16 June 2022
Published 18 June 2022

ABSTRACT

Laboratory and pot culture experiments were carried to study the effect of different bioagents, organic amendments, silver nanoparticles and fungicide in management of crossandra wilt caused by *Fusarium incarnatum* and nematode *Pratylenchus. delattrei* During the study observations are made on changes in activity of phenylalanine ammonia lyase (PAL), phenols and β 1, 3 Glucanase in crossandra plants. All the treatments applied recorded increased levels of phenylalanine ammonia lyase (PAL), phenols and β 1, 3 Glucanase in treated plants, but among all soil application (SA) of *T.viride* @ 2.5 kg/ha at 20 DAP plus soil drenching (SD) of carbendazim @ 0.1% at 30 DAP plus SA of *T.viride* @ 2.5 kg/ha at 50 DAP plus Foliar application (FA) of *P. fluorescens* @ 1.0 kg/ha at 70 DAP plus Foliar application (FA) of *B. subtilis* @ 1.0 kg /ha at 90 DAP was found to be significantly higher in induction of phenylalanine ammonia lyase (PAL), phenols and β 1, 3 Glucanase in crossandra. The same results are also noticed in later stage in reducing disease incidence compare to control and other treatments.

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Keywords: Crossandra; Fusariu spp; phenylalanine ammonia lyase; phenols; β 1; 3 Glucanase.

1. INTRODUCTION

Crossandra (Fire cracker) is an important commercial flower, mainly grown in India, Tropical Africa and Madagascar [1]. Crossandra belongs to the family of Acanthaceae and Native of Southern India and Sri Lanka. It is an erect, evergreen sub shrub growing to 1 meter and may appear at any time throughout the year and easy to grow. It can be cultivated very conveniently by small farmers.It is popularly known as Kanakambaram in Tamil, Malayalam and Telugu and Kanakambara in Kannada. In Maharashtra it is known as Aboli. There are 50 species but only a few like *Crossandra infundibuliformis, C. guineensis, C. nilotica, C. muronata* and *C. subacaulis* are cultivated.

"The flowers are commonly used for hair adornment. Though not fragrant, these flowers are very popular because of their attractive bright colour, light weight and good keeping quality. The flowers are used for making garlands, either alone or in combination with jasmine flowers. Steady market demand as well as assured and regular income have made crossandra a profitable venture for south Indian farmers" [2].

"Crossandra (*Crossandra infundibuliformis*) is affected by different fungal, bacterial, nematode and viral diseases. Among the various fungal diseases wilt disease caused by *Fusarium spp.* is one of the major problem in Crossandra production and limits the crop cultivation and it is also always associated with nematode such as *Pratylenchus delettrei"* [3]. Management of this disease has become very difficult due to its soil borne and complex nature.

Integrated approach for the management of disease involves the use of two or more methods in a compatible manner to reduce disease incidence with minimal hazards on environment and maximization of economic profit. Biocontrol agents, organic amendments, chemicals and more recently nanoparticles etc, can be combined to develop an efficient, eco-friendly, compatible and profitable disease management strategy that conserves natural resources and beneficial microbes. In all the methods of disease control, activation of defense mechanisms are very important for effective disease management. Kamalakannan [4] reported that "soil application of bio control agents such as *Trichoderma* species and bacterial isolates like *P. fluorescens* induced coleus plants to synthesize more amount of Peroxidase, Polyphenol oxidase, Phenylalanine ammonia - lyase and total phenols. PAL is the key enzyme in inducing synthesis of salicylic acid (SA) which induces systemic resistance in many plants". "Seed treatment and seedling root dipping with PGPR induced early and enhanced levels of PAL in rice plants" [5]. "When pathogens attack, phenolic compounds are produced which are considered as a part of the active defense response in plants" [6]. "Metabolomics studies revealed the involvement of phenolic compounds in plant-pathogen interactions" [7,8].

Hence, the present research was planned to study the role of defense enzymes in crossandra wilt management under pot and laboratory experiments with different treatments and their effect on disease incidence in crossandra plants.

2. MATERIALS AND METHODS

The local crossandra cultivar growing in the Madurai area was used in all pot culture experiments. The pathogen (*F. incarnatum*) mass multiplied on sand maize medium was incorporated in the pots at 3 per cent (w/w) and nematode was inoculated @ 1 nematode per gram of soil. All the treatments like biagents, nanoparticles, organic amendments and chemicals were applied as per schedule. The observations on enzyme studies were carried out after last application as mentioned in the treatment schedule. The leaves were collected from the pots 0, 3, 5, 7 and 9 days after last application in each treatment separately and washed several times with sterile distilled water before enzyme extraction. Each treatment was replicated six times with each replication containing three pots with two plants in each pot. The treat mental details are as follow in List 1.

2.1 Assay of Phenyl Alanine Ammonia Lyase (PAL)

Five hundred mg of leaf collected from each treatment was homogenized in five ml of cold 25 mM borate HCl buffer (pH 8.8) containing 5 mM mercaptoethanol, 0.4 ml per liter separately. The homogenate was centrifuged at 15,000 rpm for 15 minutes and the supernatant obtained from the samples of each treatment was used as enzyme source separately. The assay mixture consists of 0.2 ml of enzyme extract,

List 1. The treatments

T_1	SA of *P.fluorescens* (Pf-18) @ 2.5 kg/ha at 20 DAP + Module A
T_2	SA of *T.v* (Tv-9) @ 2.5 kg/ha at 20 DAP + Module A
T_3	SA of *B. subtilis* (Bs-10) @2.5 kg/ha at 20 DAP + Module A
T_4	SA of Neem cake @ 250 kg/ha at 20 DAP + Module A
T_5	SD of carbendazim @ 0.1% at 20 DAP + Module A
T_6	SA of Phorate10G @10 kg/ha at 20DAP + Module A
T_7	FA of nano particles @ 800 ppm at 20 DAP + Module A
T_8	SA of *P.f* (Pf-18) @ 2.5 kg/ha at 20 DAP + Module B
T_9	SA @ *T.v* (Tv-9) @ 2.5 kg/ha at 20 DAP + Module B
T_{10}	SA of *B.s* (Bs-10) @ 2.5 kg/ha at 20 DAP + Module B
T_{11}	SA of Neem cake @ 250 kg/ha at 20 DAP + Module B
T_{12}	SD of carbendazim @ 0.1% at 20 DAP + Module B
T_{13}	SA of Phorate10G @10 kg/ha at 20DAP + Module B
T_{14}	FA of nano particles @ 800 ppm at 20 DAP + Module B
T_{15}	SA of *P.f* (Pf-18) @ 2.5 kg/ha at 20 DAP + Module C
T_{16}	SA *T.v* (Tv-9) @ 2.5 kg/ha at 20 DAP + Module C
T_{17}	SA of *B.s* (Bs-10) @ 2.5 kg/ha at 20 DAP + Module C
T_{18}	SA of Neem cake @ 250 kg/ha at 20 DAP + Module C
T_{19}	SD of carbendazim @ 0.1% at 20 DAP + Module C
T_{20}	SA of Phorate10G @10 kg/ha at 20DAP + Module C
T_{21}	FA of nanoparticles @ 800 ppm at 20 DAP + Module C
T_{22}	Inoculated control (Pathogens and nematode)
T_{23}	Un inoculated control (No Pathogens and nematode)

Module A.= Foliar application (FA) of nano particles @ 800ppm at 30 DAP, SA of T. viride (Tv-9) @ 2.5 kg/ha at 50 DAP + FA of P.f (Pf-18) @ 1.0 kg/ha at 70 DAP + FA of of B.s (Bs-10) @ 1.0 kg/ha at 90 DAP
Module B = FA of carbendazim @ 0.1% at 30 DAP + SA of T.v @ 2.5 kg/ha at 50 DAP and FA of P.f (Pf-18) @ 1.0 kg/ha at 70 DAP + FA of B.s (Bs-10) @ 1.0 kg/ha at 90 DAP
Module C= SD of carbendazim @ 0.1% at 30 DAP + SA of T.v @ 2.5 kg/ha at 50 DAP + FA of P.f (Pf-18) @ 1.0 kg/ha at 70 DAP + FA of B.s (Bs-10) @ 1.0 kg/ha at 90 DAP

1.3 ml water and 0.5 ml borate buffer. The reaction was initiated by the addition of one ml of 12 mM L-Phenylalanine. The reaction mixture was incubated for one h at 32˚C. The reaction was stopped by the addition of 0.5 ml of 2N HCl. A blank was run in which phenylalanine was added after adding 2N HCl. The absorbance was measured at 290 nm. The enzyme activity was expressed as μmol of cinnamic acid/ minute/ g of leaf [9].

2.2 Assay of Phenols

One g of the leaf sample was ground in a pestle and mortar in 10 ml of 80 per cent methanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was evaporated to dryness and the residue was dissolved in five ml of distilled water. From this, 0.2 ml was taken and the volume was made up to three ml with distilled water. To that 0.25 ml of (1N) Folin-Ciocalteau reagent was added. After three minutes, one ml of 20 per cent sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for one minute and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed as μg of catechol g^{-1} of leaf tissue [10].

2.3 Assay of β-1, 3- glucanase

β-1, 3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method [11]. The reaction mixture was consisted of 62.5μl of four per cent laminarin and 62.5 μl of enzyme extract. The reaction was carried out at 40°C for 10 minutes. The reaction was then stopped by adding 375 μl of dinitrosalicylic acid and heated for five minutes on boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as μg glucose released min^{-1} mg^{-1} of protein.

3. RESULTS AND DISCUSSION

Changes in phenylalanine ammonia lyase (PAL) activity in plants: In crossandra plants after treating with different combinations of bio agents, organic amendments, nanoparticles and

chemicals the activity of PAL was induced in all the treated plants which were challenge inoculated with the pathogens and nematode. The enzyme activity reached the maximum at five days after last application and maintained at higher level up to seven days after application and slowly declined thereafter in all the treatments. Whereas in healthy and pathogen and nematode inoculated control plants the activity was less than that in all the other treatments. The treatment T_{16} was found to record higher activity of PAL (0.895 µ mol of transcinnamic acid / min /g of leaf tissue) (Table 1).

"Phenyl propanoid metabolism starts with the conversion of L-phenylalanine into trans-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) and supplies the precursors for flavanoid pigments, lignins and phytoalexins" [12,13]. "Increase in PAL activity subsequently increased the phenolic contents and leads to disease resistance. In the present study, treatment with integrated module T_{16} induced the plants to synthesize PAL when they were challenge inoculated with *F.incarnatum* and *P. delattrei*. When turmeric plants were sprayed with *P. fluorescens* isolates, PAL activity increased" [14,15]. "Phenylalanine ammonia lyase played an important role in the biosynthesis of phenolic phytoalexins" [16]. "The product of PAL activity was transcinnamic acid which was an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (SAR)" [17]. "Cell wall strengthening, through the deposition of lignin, proceeded by the induction of the synthesizing

Table 1. Changes of phenylalanine ammonia lyase (PAL) activity in crossandra plants treated with integration of different combinations of bio agents, organic amendments, nanoparticles and chemicals on the management of wilt in pot culture experiment

T. No.	Name of the treatment	µ mol of transcinnamic acid / min /g of leaf tissue*					% Disease incidence
		Days after last application					
		0	3	5	7	9	
1	T_1	0.472	0.684	0.878	710	685	58.3(49.79)**
2	T_2	0.485	0.702	0.884	0.750	0.695	50.0(44.98)
3	T_3	0.479	0.682	0.870	0.706	0.680	61.1(51.41)
4	T_4	0.486	0.658	0.846	0.691	0.652	63.9(53.06)
5	T_5	0.478	0.690	0.878	0.734	0.682	52.8(46.58)
6	T_6	0.483	0.694	0.880	0.742	0.688	63.9(53.06)
7	T_7	0.492	0.692	0.884	0.745	0.680	55.6(48.18)
8	T_8	0.472	0.680	0.874	0.706	0.674	36.1(36.92)
9	T_9	0.479	0.695	0.875	0.700	0.678	30.6((33.54)
10	T_{10}	0.473	0.682	0.864	0.698	0.673	36.1(36.92)
11	T_{11}	0.474	0.655	0.840	0.685	0.642	44.4(41.79)
12	T_{12}	0.471	0.688	0.874	0.730	0.680	33.3(35.24)
13	T_{13}	0.472	0.692	0.870	0.740	0.675	41.7(40.18)
14	T_{14}	0.479	0.690	0.881	0.743	0.671	36.1(36.92)
15	T_{15}	0.468	0.688	0.883	0.712	0.689	27.8(31.79)
16	T_{16}	0.470	0.712	0.895	0.790	0.712	16.7(24.08)
17	T_{17}	0.472	0.685	0.875	0.693	0.678	19.4(26.15)
18	T_{18}	0.481	0.660	0.852	0.695	0.654	22.2(28.11)
19	T_{19}	0.473	0.692	0.885	0.742	0.690	16.7(24.08)
20	T_{20}	0.468	0.702	0.888	0.754	0.695	19.4(26.15)
21	T_{21}	0.475	0.694	0.882	0.748	0.687	22.2(28.11)
22	T_{22}	0.480	0.524	0.533	0.520	0.521	75.0(60.04)
23	T_{23}	0.484	0.498	0.499	0.482	0.480	**2.73(0.627)**

** Mean of three replications, DAP – Days after planting, CD (P=0.05), Treatments 0.03, Days 0.01, Treatments x Days 0.06*

Module A.= Foliar application (FA) of nano particles @ 800ppm at 30 DAP + SA of T. viride (Tv-9) @ 2.5 kg/ha at 50 DAP + FA of P.f (Pf-18)@ 1.0 kg/ha at 70 DAP + FA of of B.s (Bs-10) @ 1.0 kg/ha at 90 DAP

Module B = FA of carbendazim @ 0.1% at 30 DAP + SA of T.v (Tv-9) @ 2.5 kg/ha at 50 DAP + FA of P.f (Pf-18) @ 1.0 kg/ha at 70 DAP + FA of B.s @ 1.0 kg/ha at 90 DAP

Module C= SD of carbendazim @ 0.1% at 30 DAP + SA of T.v (Tv-9) @ 2.5 kg/ha at 50 DAP + FA of P.f (Pf-18)@ 1.0 kg/ha at 70 DAP + FA of B.s (Bs-10) @ 1.0 /ha at 90 DAP

enzymes were played an important role in the defense response of *Lycopersicon esculentum* in reaction to a variety of elicitors" [18].

3.1 Changes in phenols and β 1, 3 Glucanase activities in Crossandra Plants

The accumulation of phenol was found to increase from the third day after treatment and attained a peak on fifth days after last application and thereafter slowly declined. The treatment T_{16} recorded maximum total phenol content (668.02 μg of catchol/ g leaf tissue) at 5 days after last application whereas no significant change in the phenol content was observed in the uninoculated control (Table 2).

β 1, 3 glucanase activity was at a lower level in the uninoculated control. The treatment T_{16} was found to record high enzyme activity (57.43 μg of glucose / min /g of leaf tissue) at 5 days after last application and it was followed by T_2 which recording (56.43 μg of glucose / min /g of leaf tissue) at 5 days after last application. In the inoculated control the enzyme activity was found to increase slightly (Table 3).

Many phenols and their oxidative products such as quinones were highly toxic to the invading pathogens [19] and enhanced the mechanical strength of host cell wall. The results of the present study demonstrated that higher levels of phenol accumulation were observed in the T_{16} treated crossandra plants. Similar findings were reported in rice against *R. solani* [15,20], sugarcane against *Colletotrichum falcatum* [21], groundnut against *Cercospora personata* [22], and turmeric against *Pythium aphanidermatum* [14]. Raj Kumar [23] reported that the increase in phenol content in banana plants upto five days when treated with combined biocontrol agent aplication of BBs_1+BBs_2+ BPf_1.

Table 2. Changes of phenol activity in crossandra plants treated with integration of different combinations of bio agents, organic amendments, nanoparticles and chemicals on the management of wilt in pot culture experiment

T. No.	Name of the treatment	μg of catachol /g of leaf tissue*					%Disease incidence
		Days after last application					
		0	3	5	7	9	
1	T_1	524.45	595.54	611.12	605.44	600.45	58.3(49.79)**
2	T_2	521.31	630.82	664.97	662.87	657.46	50.0(44.98)
3	T_3	532.03	593.50	608.06	601.77	598.42	61.1(51.41)
4	T_4	524.45	572.68	594.40	590.55	586.3	63.9(53.06)
5	T_5	525.32	610.20	648.52	608.33	605.46	52.8(46.58)
6	T_6	522.78	620.45	655.60	650.30	645.22	63.9(53.06)
7	T_7	525.21	612.80	650.77	645.22	641.58	55.6(48.18)
8	T_8	525.73	590.23	605.32	601.88	595.64	36.1(36.92)
9	T_9	520.95	592.34	660.35	658.75	598.54	30.6((33.54)
10	T_{10}	521.31	592.34	604.96	600.22	596.24	36.1(36.92)
11	T_{11}	522.54	570.86	590.06	588.43	584.08	44.4(41.79)
12	T_{12}	524.28	608.70	644.32	602.66	602.46	33.3(35.24)
13	T_{13}	525.31	615.34	652.85	648.55	643.44	41.7(40.18)
14	T_{14}	523.53	610.83	645.87	640.32	634.52	36.1(36.92)
15	T_{15}	524.93	530.55	598.45	620.18	610.74	27.8(31.79)
16	T_{16}	522.01	635.94	668.02	660.26	668.94	16.7(24.08)
17	T_{17}	524.45	598.42	612.85	611.75	605.24	19.4(26.15)
18	T_{18}	525.32	576.74	598.44	596.53	588.6	22.2(28.11)
19	T_{19}	522.78	612.40	652.25	613.37	608.64	16.7(24.08)
20	T_{20}	525.21	621.38	658.60	653.66	647.33	19.4(26.15)
21	T_{21}	525.73	615.08	653.88	649.22	645.83	22.2(28.11)
22	T_{22}	520.95	524.44	546.81	533.48	526.45	75.0(60.04)
23	T_{23}	521.31	521.15	526.84	521.38	519.10	**2.73(0.627)**

** Mean of three replications, DAP – Days after planting, CD (P=0.05), Treatments 0.03, Days 0.01, Treatments x Days 0.06*

Table 3. Changes of β 1, 3 Glucanase activity in crossandra plants treated with integration of different combinations of bio agents, organic amendments, nanoparticles and chemicals on the management of wilt in pot culture experiment

T. No.	Name of the treatment	µg of glucose / min /g of leaf tissue*					% Disease incidence
		Days after last application					
		0	3	5	7	9	
T_1	T_1	38.09	43.12	53.37	52.74	50.81	58.3(49.79)**
T_2	T_2	37.23	45.67	56.43	54.34	52.72	50.0(44.98)
T_3	T_3	36.67	42.21	52.48	51.87	50.34	61.1(51.41)
T_4	T_4	37.36	41.96	51.12	50.28	49.13	63.9(53.06)
T_5	T_5	36.53	44.67	55.55	53.43	51.92	52.8(46.58)
T_6	T_6	37.02	45.09	53.45	53.23	52.34	63.9(53.06)
T_7	T_7	38.17	43.59	53.42	52.85	50.93	55.6(48.18)
T_8	T_8	38.12	42.21	52.77	51.75	50.12	36.1(36.92)
T_9	T_9	38.02	44.63	55.60	53.21	51.60	30.6((33.54)
T_{10}	T_{10}	37.94	41.35	51.80	50.94	50.15	36.1(36.92)
T_{11}	T_{11}	37.13	41.24	50.74	49.88	48.05	44.4(41.79)
T_{12}	T_{12}	36.98	43.61	52.88	52.21	51.30	33.3(35.24)
T_{13}	T_{13}	37.64	44.54	53.08	53.62	51.96	41.7(40.18)
T_{14}	T_{14}	37.26	43.14	51.67	51.98	50.23	36.1(36.92)
T_{15}	T_{15}	37.36	44.45	54.82	53.74	51.93	27.8(31.79)
T_{16}	T_{16}	36.53	46.60	57.43	55.38	52.72	16.7(24.08)
T_{17}	T_{17}	37.02	43.46	53.84	52.87	51.70	19.4(26.15)
T_{18}	T_{18}	37.94	42.64	52.14	51.30	50.13	22.2(28.11)
T_{19}	T_{19}	38.17	45.54	52.14	51.33	52.90	16.7(24.08)
T_{20}	T_{20}	36.67	46.23	53.84	52.41	51.44	19.4(26.15)
T_{21}	T_{21}	37.36	44.55	53.41	52.76	49.91	22.2(28.11)
T_{22}	T_{22}	36.53	39.36	44.96	43.92	41.72	75.0(60.04)
T_{23}	T_{23}	37.02	38.9	38.72	38.53	38.21	**2.73(0.627)**

** Mean of three replications, DAP – Days after planting, CD (P=0.05), Treatments 0.03, Days 0.01, Treatments × Days 0.06*

Different types of pathogens and abiotic stress have been found to induce pathogen related (PR) and host-coded proteins. Synthesis and accumulation of PR proteins play an important role in plant defense system [24]. The enzyme act upon the fungal cell wall, resulting in degradation and loss of inner content of cells [25].

Maurhofer et al. [25] also reported the induction of β-1, 3-glucanase and induction of systemic resistance by *P. fluorescens* strain CHAO in tobacco. Bargabus et al. [26] reported that, *B. pumilus* isolates 203-6 and 203-7 induced β-1, 3-glucanase in sugar beet. The present investigation confirms the earlier work of Xue et al. [27] who reported that the inoculation of root rot pathogen *Rhizoctonia* spp. or anthracnose pathogen *Colletotrichum lindimutheanum* induced systemic resistance by induction of peroxidases, β, 1-3-glucanases in the bean plants [28].

4. CONCLUSION

It was found that soil application (SA) of *T.viride* @ 2.5 kg/ha at 20 DAP plus soil drenching (SD) of carbendazim @ 0.1% at 30 DAP plus SA of *T.viride* @ 2.5 kg/ha at 50 DAP plus Foliar application (FA) of *P. fluorescens* @ 1.0 kg/ha at 70 DAP plus Foliar application (FA) of *B. subtilis* @ 1.0 kg /ha at 90 DAP was found to be higher in induction of phenylalanine ammonia lyase (PAL), phenols and β 1, 3 Glucanase in crossandra plants so that offering resistance in later stage in preventing disease incidence.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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