Metabolites change in *Jatropha* plants due to seed treatment with rhizobacteria and *Rhizoctonia bataticola*

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Abstract. An experiment on the metabolite [salicylic acid (SA), jasmonic acid (JA), hydrocyanic acid (HCN) and chitinase activity] changes owing to seed treatment with pathogen, plant growth promoting rhizobacteria (PG-PRs) - (*P. maltophilia, P. fluorescens* and *Bacillus subtilis*) alone and in combination was conducted at Chaudhary Charan Singh, Haryana Agricultural University, Regional Research Station, Bawal. *Jatropha curcas* plants raised from root rot pathogen (*Rhizoctonia bataticola*) treated seeds showed an initial increase in SA and hydrocyanic acid HCN content and an opposite trend was observed for JA level and chitinase activity. Though, PGPRs inoculation resulted in higher increase in SA level, JA level and chitinase activity in both the cases alone as well as in integration with pathogen, however, maximum increase in JA content was explicited in plants raised after seed treatment with *P. fluorescens*, the most effective rhizobacteria amongst PGPRs studied. Highest increase in HCN content (45 µg g^{-1}) over control (24 µg g^{-1}) was noticed for *P. fluorescens* followed by co-seed inoculation with *P. fluorescens* + pathogen (43 µg g^{-1}) at 10 DPI. The co-seed inoculation elicited 68 units at 10 DPI whereas the pathogen challenged plants showed lower chitinase activity with 42 units. All the metabolites declined slightly or sharply with age of the plant irrespective of inoculations.
Keywords metabolites, *Jatropha curcas*, rhizobacteria, *Rhizoctonia bataticola*.

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Introduction

Physic nut (*Jatropha curcas* L.) locally known as 'Ratanjot' belongs to family Euphorbiaccae. It is a large shrub or small tropical tree widely distributed in arid and semi arid areas.

It is the main commodity source for bio-diesel in India. Recently, the economic importance of Jatropha has increased because of the use of its oil as a fuel (diesel) substitute. It is truly a multipurpose tree species fit for agroforestry and other afforestation program. The root rot disease caused by *Rhizoctonia. bataticola* (Taub.) Butler has been recorded in causing 10-12 per cent mortality of 20-30 days old seedlings of Jatropha at Haryana Agricultural University, Regional Research Station, Bawal (Sharma & Kumar 2010). The incidence of this disease has been observed from other parts of Haryana too. Plant can be induced to develop enhanced resistance to pathogen infection by treatment with a variety of abiotic and biotic inducers including Plant Growth Promoting Rhizobacteria (PGPRs). The secondary metabolites viz., HCN, chitinase, signal transducers (salicylic acid and jasmonic acid) elevated during infection serve as anti-metabolites to pathogen for checking their spread inside the plant through induced systemic resistance (ISR) or systemic acquired resistance (SAR). Elicitation of resistance in plants by biocontrol agents is becoming a more researched topic. Some strains of bacterial agent like *Pseudomonas* are clearly potent inducers of systemic resistance responses in plants (Van Weas et al. 1989, Meena et al. 2000). Keeping in view the importance of plant, the resulting losses due to disease and inadequate literature, an attempt was made to elucidate the resistance mechanisms by estimating biochemical constituents.

Material and methods

The experiment was conducted in earthen pots having 5 kg sterilized soil. The biocontrol agents *viz.*, *Pseudomonas fluorescens*, *P. maltophilia* and *B. subtilis* were grown in nutrient broth (peptone 5 g, yeast extract 2 g, sodium chloride 5 g, beef extract 1 g, distilled water 1000 ml) and the pathogen (*R. bataticola*) on potato dextrose agar (potato infusion 200 g, dextrose 20 g, agar 20 g, distilled water 1000 ml). For each treatment, the seeds were surface sterilized with 2 per cent sodium hypochlorite solution for half a minute and then washed 2-3 times with sterilized distilled water. The seeds were coated with slurry of mycelial mat of the pathogen. The required quantity of mycelial mat (15 g/kg seed) was taken in a container and sufficient quantity of water was added to make it as a thin paste but not too watery. The seed was mixed thoroughly with paste for even distribution of the pathogen. Suitable sticker like carboxy methyl cellulose (0.1%) was added for better adherence of pathogen to the seed. The seed was dried in shade before sowing (Kumar et al. 2011). In case of inoculation with PGPRs + pathogen, the seeds were treated first with bio-agents (dipping in suspension of 1×10^8 cfu/ml for half an hour) and air dried followed by coating with mycelial mat of pathogen and again air dried to ensure the infection. The seeds coated with *R. bataticola* and three PGPRs individually served as control. After seed treatment, 15 seeds were sown in each pot with three replications. The pots were watered regularly. The Jatropha plants were raised as per treatments: 1) Seed inoculation with *P. maltophilia*; 2) Seed inoculation with *P. fluroscens*; 3) Seed inoculation with *B. subtilis*; 4) Seed inoculation with *R. bataticola* (pathogen); 5) Co-seed inoculation with *P. maltophilia* + *R. bataticola*; 6) Co-seed inoculation with *P. fluroscens* + *R. bataticola*; 7) Co-seed inoculation with *B. subtilis* + *R. bataticola*; 8) Control (untreated). The root samples were collected from uninoculated (control) and inoculated plants at 10th, 20th, 30th, 40th and 50th days of inoculation. The sample required for enzymatic studies were collected in plastic bags and deep frozen until used. Quantitative assay of salicylic acid (SA) was carried out by method of Meyer & Hofte (1997). The samples (roots) for each parameter were collected at different intervals i.e. after 10, 20, 30, 40 and 50 days of inoculation and also from the healthy (untreated) plants. The

Method of Schittko et al. (2000) were used for extraction, purification and determination of jasmonic acid (JA). Nehra et al. (1994) method was used for chitinase enzyme. Hydrocyanic acid (HCN) content in Jatropha plant was assayed by method of Lee et al. (1993). Statistical analysis of data was done on the basis of angular transformed values of per cent disease (George & Willium, 1957).

Results

Salicylic acid. Change in salicylic acid was evaluated in Jatropha after biotic stress. The results accentuated in Table 1 indicated no differences in salicylic acid level in *P. moltophila*, *B. subtilis* treated plants and the control. It ranged from 19-20 µM. However, there was slight decline with plant age. *P. fluorescens* treated plants showed a 105 per cent increase in SA level (37 µM) over control at 10 DPI. Challenging the plant with pathogen showed

a sharp increase in SA level at 10 DPI (108 µM). Yet it also sharply declined and remained only 18 µM at 50 DPI. This value was slightly higher over the control (14 µM) at 50 DPI. Co-seed inoculation with *P. fluorescens* + pathogen was adjudged the most effective treatment as it showed the highest SA level (126 µM). *P. maltophilia* + pathogen and *B. subtilis* + pathogen showed also significant differences in SA level over the control.

Jasmonic acid. Change in Jasmonic acid content was evaluated in Jatropha after biotic stress and the results given in Table 2 indicated significant differences in JA level in *P. moltophila*, *P. fluorescens*, *B. subtilis* treated plant over the control. It ranged from 1.35-1.64 µg as compared to 0.34 µg in control. There was a slightly decline as the plant aged. *P. fluorescens* treated plants showed five fold increase in JA level (1.64 µg) over the control. Further, challenging the plant with pathogen alone showed decrease in JA level at 10 DPI (0.28 µg). But as the time proceeded, the decline was gradual

Table 1 Change in salicylic acid in *Jatropha* roots due to seed treatment with PGPRs and/or inoculation with *Rhizoctonia bataticola*

| Treatment | Concentration of salicylic acid (µM ml^{-1} enzyme extract) | | | | |
| | Days Post Inoculation (DPI) | | | | |
	10	20	30	40	50
Pseudomonas maltophilia	20* (2.94)** 11.11	18 (1.73) 5.88	16 (1.63) 6.66	15 (2.93) 0.00	14 (1.25) 0.00
Pseudomonas fluorescens	37 (2.05) 105.5	33 (0.82) 94.11	31 (0.82) 106.67	28 (1.63) 86.67	26 (1.41) 85.71
Bacillus subtilis	19 (0.82) 5.55	17 (1.41) 0.00	16 (0.82) 6.67	16 (0.82) 6.67	15 (2.05) 7.14
Rhizoctonia bataticola	108 (1.41) 500	68 (2.16) 300	46 (3.74) 206.67	22 (2.49) 46.67	18 (2.05) 28.57
P. maltophilia + *R. bataticola*	107 (2.94) 494	65 (1.70) 282.35	45 (1.42) 200.00	20 (2.05) 33.33	17 (1.63) 21.43
P. fluorescens + *R. bataticola*	126 (3.09) 600	81 (2.94) 326.47	61 (2.94) 306.67	37 (1.70) 146.67	29 (2.05) 107.14
B. subtilis + *R. bataticola*	110 (1.63) 511	63 (2.16) 270.59	42 (2.49) 180.00	21 (2.16) 40.00	16 (1.70) 14.29
Control	18 (2.83)	17 (1.63)	15 (1.63)	15 (1.70)	14 (1.25)

Note. * Values are mean of three replicates. In parentheses is the standard deviation. The bellow values are the per cent increase after control.

Table 2 Change in jasmonic acid in *Jatropha* roots due to seed treatment with PGPRs and /or inoculation with *R. bataticala*

Treatment	Concentration of Jasmonic acid (µg ml⁻¹ enzyme extract)				
	Days of inoculation				
	10	20	30	40	50
Pseudomonas maltophilia	1.48 (0.81)	1.12 (1.47)	0.93 (1.23)	0.72 (0.27)	0.53 (0.41)
	335.23	239.14	190.71	140.32	96.50
Pseudomonas fluorescens	1.64 (0.06)	1.30 (0.81)	1.07 (0.27)	0.81 (0.82)	0.62 (1.25)
	384.12	294.13	234.20	170.80	129.21
Bacillus subtilis	1.35 (1.70)	1.09 (1.63)	0.87 (0.47)	0.69 (1.63)	0.47 (2.05)
	297.17	230.42	172.18	130.70	74.39
R. bataticola	0.28 (0.94)	0.26 (0.82)	0.25 (1.25)	0.23 (1.63)	0.20 (0.94)
	-17.64	-21.21	-21.86	-23.23	-25.93
P. maltiphila + *R. bataticola*	1.36 (2.05)	1.08 (0.47)	0.86 (1.73)	0.65 (1.63)	0.46 (2.93)
	300	227.27	168.75	116.67	70.37
P. fluorescens + *R. bataticola*	1.52 (1.63)	1.24 (2.49)	0.99 (2.16)	0.74 (1.70)	0.57 (2.05)
	347.06	275.75	190.63	146.67	111.11
B. subtilis + *R. bataticola*	1.23 (1.73)	0.98 (2.83)	0.78 (1.41)	0.61 (3.09)	0.38 (0.94)
	261.76	196.97	143.75	103.33	40.75
Control	0.34 (0.08)	0.33 (1.63)	0.32 (1.70)	0.30 (1.25)	0.27 (0.82)

Note. In parentheses is the standard deviation. The bellow values are the per cent increase after control.

as at 50 DPI. This value was recorded as 0.20 µg. The most effective treatment having co-inoculation (*P. fluorescens* + pathogen) showed the highest JA level (1.52 µg) while *P. maltophilia* + pathogen and *B. subtilis* + pathogen showed 1.36 µg and 1.23 µg, respectively.

Chitinase. Results depicted in Table 3 revealed similar trends of JA were observed for chitinase activity. It ranged from 16.67 to 88.89 per cent at 10 DPI and 3.23 to 77.41 per cent at 50 DPI over the control. Seed inoculation with PGPRs alone showed a higher increase in chitinase activity. *P. fluorescens*, the most effective treatment amongst PGPRs, showed 55 units at 10 DPI (one Units = 1.0 µM of NAG min⁻¹ g⁻¹ fresh weight) over the control (36 units). Pathogen challenged plant depicted a low chitinase activity with 42 units at 10 DPI. Co-seed inoculation showed further increase in chitinase activity over PGPRs alone. *P. fluorescens* + pathogen showed 68 units at 10 DPI and 55 units at 50 DPI followed by *B. Subtilis* + pathogen and *P. maltophilia* + pathogen with 62 and 61 units at 10 DPI, respectively. There was a negative correlation between plant age

and chitinase activity.

Hydrocyanic acid. Results revealed that *P. fluorescens* the most effective treatment, showed a highest increase in HCN content (45 µg) over control (24 µg) followed by co-seed inoculation with *P. fluorescens* + pathogen (43 µg) at 10 DPI (Table 4). *P. fluorescens* showed 72-91 per cent increase in HCN content at different stages of sampling. Seed bacterization with *P. maltophilia, B. subtilis* were statistically at par with the control. Jatropha plant raised from pathogen treated seed showed an initial increase in HCN content (34 µg) over the control (24 µg) and later sharply declined as plant grown older (12 µg). Further analysis revealed that pathogen treated plant showed 33.33 per cent decrease in HCN content over control (18 µg) at 50 DPI. No additive effect was observed in co-seed inoculation with *P. maltophilia* + pathogen and *B.subtilis* + pathogen. In *P. maltophilia* + pathogen treatment, HCN content was 37 µg at 10 DPI and declined with plant age (16 µg at 50 DPI). Similarly, *B. subtilis* + pathogen showed 36 µg HCN content at 10 DPI and declined with plant age (14 µg).

Table 3 Effect of seed treatment with PGPRs and/or *R. bataticola* on chitinase activity of *Jatropha* plant

| Treatment | Chitinase activity (one unit - 1.0 µM of NAG min^{-1} g^{-1} fresh tissue) | | | | |
| | Days Post Inoculation (DPI) | | | | |
	10	20	30	40	50
Pseudomonas	48 (1.25)	45 (1.41)	42 (1.63)	40 (1.70)	38 (1.25)
maltophilia	33.33	28.57	27.27	25.00	22.58
Pseudomonas	55 (0.47)	53 (1.70)	49 (1.25)	47 (0.94)	45 (1.25)
fluorescens	52.78	51.42	48.48	46.88	45.16
Bacillus	50 (1.25)	48 (0.47)	45 (2.49)	42 (1.70)	40 (1.63)
subtilis	38.89	37.14	36.36	31.25	29.03
Rhizoctonia	42 (1.25)	39 (1.25)	36 (1.25)	34 (1.70)	32 (0.47)
bataticola	16.67	11.43	9.09	6.25	3.23
P. maltophilia	61 (0.94)	59 (1.70)	54 (0.47)	52 (1.63)	49 (1.25)
+ R. bataticola	69.44	68.57	66.67	65.63	61.29
P. fluorescens	68 (0.94)	65 (0.47)	61 (1.70)	58 (1.25)	55 (1.70)
+ R. bataticola	88.89	85.71	84.85	81.25	77.41
B. subtilis	62 (1.70)	59 (1.42)	55 (1.63)	53 (2.05)	50 (0.47)
+ R. bataticola	72.22	68.37	63.64	62.5	58.06
Control	36 (1.63)	35 (0.94)	33 (1.70)	32 (0.94)	31 (0.82)

Note. In parentheses is the standard deviation. The bellow values are the per cent increase after control. NAG - N-acetyl glucosamine.

Table 4 Change in HCN content in *Jatropha roots* due to seed treatment with rhizobacteria and/or pathogen

| Treatment | HCN content(mg/g fresh weight) | | | | |
| | Days Post Inoculation (DPI) | | | | |
	10	20	30	40	50
Pseudomonas	29* (1.25)**	27 (1.70)	25 (1.41)	23 (1.70)	20 (2.05)
maltophilia	20.83	22.72	19.04	15.00	11.11
Pseudomonas	45 (1.25)	42 (1.42)	39 (0.82)	36 (1.25)	31 (0.82)
fluorescens	87.50	90.90	85.71	80.00	72.22
Bacillus	28 (0.94)	25 (1.70)	24 (0.82)	22 (1.25)	19 (0.47)
subtilis	16.67	13.63	14.28	10.00	5.56
Rhizoctonia	34 (1.70)	28 (1.70)	19 (0.94)	14 (0.82)	12 (1.25)
bataticola	41.67	27.27	-9.53	-30.00	-33.33
P. maltophilia	37 (0.82)	31 (2.16)	25 (0.47)	17 (0.94)	16 (0.47)
+ R. bataticola	54.17	40.90	19.05	-15.00	-11.11
P. fluorescens	43 (0.47)	39 (1.25)	37 (1.25)	34 (1.25)	29 (0.82)
+ R. bataticola	95.45	77.27	76.10	70.00	61.11
B. subtilis	36 (0.47)	30 (2.05)	21 (1.25)	17 (0.47)	14 (1.70)
+ R. bataticola	50.00	36.36	0.00	-15.00	-22.22
Control	24(0.82)	22 (1.42)	21(1.25)	20(0.47)	18(1.25)

Note. * Values are mean of three replicates. In parentheses is the standard deviation. The bellow values are the per cent increase after control.

Discussion

Salicylic acid (SA). Salicylic acid is known to play a critical signaling role in the activation of plant defence responses after pathogen attack (Klessing et al. 2000). Our result shows enhanced level of SA after inoculation with pathogen (108 μM) or with *P. fluorescens* (37 μM). However, with other two PGPRs, *P. maltophilia* and *B. subtilis*, the level of SA were 20 μM and 19 μM, respectively over the control (18 μM) at 10 DPI. Plants raised from co-inoculated seeds (*P. fluorescens* + pathogen) showed a further increase in SA level (126 μM at 10 DPI and 29 μM at 50 DPI) as compared to the infection with *R. bataticola* alone (18 and 14 μM, respectively). The results also indicated a negative correlation between SA level and plant age. It may be due to plant metabolism. Similar results have been reported in earlier findings of several other workers (Malamy et al. 1990, Maurhofer et al. 1994, Michal Shoresh et al. 2005). They observed an increase in SA level after pathogenic infection. SA production by rhizobacteria in rhizosphere may enhance defence mechanism in plants. PGPRs do not have a direct effect on SA level of plants, since PGPRs causes ISR (Jasmonic acid based resistance) not SAR (SA based resistance). Pathogen caused SAR but later it disappeared due to rapid plant metabolism. Co-seed treatment with PGPRs and pathogen caused further increase of SA level. It in not due to decline in rapid metabolism but due to uptake of SA by plant roots, produced by rhizobacteria in rhizosphere.

Jasmonic acid (JA). Jasmonic acid and its methyl ester (MeJA), collectively termed Jasmonates, are fatty acid-derived, naturally occurring octadecanoide-based compounds which are synthesized from linolenic acid by lipoxygenas (Vick & Zimmerman 1984). These compounds are not only involved in defense responses against pathogen attack (Blechert et al. 1995), but also in plant growth and development (Creelman & Mullet 1997).

In present studies, enhanced level of JA after inoculation with PGPRs and not with pathogen was noticed. JA levels in PGPRs ranged from 1.35-1.64 μg as compared to 0.34 μg in the control at 10 DPI. Plants raised from co-inoculated seeds (PGPRs + pathogen) showed slight decrease (261-347%) in JA level as compared to PGPRs alone (297-384%). It may be due to antagonistic relationship of SA and JA. SA inhibits the synthesis of JA and JA-inducible proteins (Seo et al. 1997). JA and ethylene are the signal molecules involved in ISR mediated by rhizobacteria (Michal Shoresh et al. 2005). Results also revealed a negative correlation between JA level and plant age. These results are in close conformity with earlier studies (Farmer 1994, Seo et al. 1995).

Chitinase. The increase in chitinase and 1,3-β glucanases has been previously reported to have antifungal activities in plants (Maurhofer et al. 1994). The enzymes protect plants against fungi causing a lytic action on fungal cell wall or by releasing signal compounds that may activate a variety of plant defenses (Mauch et al. 1988). In the present study, seed bacterization with PGPRs reported an increase (33-39%) in chitinase activity over the control at 10 DPI. Pathogen challenge also induced chitinase activity. Nehra et al. (1994) and Nandakumar et al. (2001) reported similar results. Chitinase activity increased in leaves and pods of resistant as well as susceptible cultivars of chickpea (*Cicer arietinum*) when inoculated with *Ascochyto rabiei* (Nehra et al. 1994). *Psendomonas fluorescens* strains have been reported to induce systemic resistance by activating chitinase genes in rice (Nandakumar et al. 2001). It is also revealed from our study that co-seed inoculation showed further increase (69-89% at 10 DPI) in chitinase activity over PGPRs alone (33-53% at 10 DPI) over the control. These observations were in agreement with Benhamou et al. 1996 and M'Piga et al. 1997. Benhamou et al. (1996) reported more chitinase with *P. fluorescens* inoculation in pea roots at the site of *Fusarium oxysporum*

f.sp. *pisi* penetration. A two-fold increase in chitinase activity occurred two days after inoculation of rice plants with the pathogen. (Radja Commere et al. 2004).

Hydrocyanic acid (HCN). Seed bacterization with *Pseudomonas fluorescens* enhanced the HCN content in Jatropha plant. This increase was two fold than the control (24 µg g^{-1} fresh weight). Voisard et al. (1989) and Bhatia et al. (2005) also reported that *P. fluorescens* produce HCN content in rhizosphere, which is absorbed by the Plant resulting in suppression of disease incidence. Ping wing (1992) reported that a cynogenic plants proposed rapid generation of HCN as a general defence against animals, insects, microorganisms and abiotic stress. Similar results were found in our studies. Plant challenged with pathogen show a rapid increase (41.67%) in HCN content at 10 DPI over the control. However, it later declined sharply. This is in the line of order of finding of Ping Wing et al. (1992). They reported that pathogen produce an enzyme cyanide hydratase (CHT) which converts the HCN to formamide. Among the PGPRs, *P. maltophilia* and *B. subtilis* do not produce HCN (Castric & Castric 1983), therefore, act as microorganism and showed non-significant difference with control. In case of co-seed inoculation, PGPRs first interact with pathogen and then induce effect on HCN content. The co-seed inoculation with *P. fluorescens* + pathogen showed a higher increase (95.4%) in HCN content, but lower than *P. fluorescens* alone (87.5%). It may be due to physical interaction between them and resulted in population reduction. Bhatia et al. (2005) reported that seed bacterization with strains of fluorescent *Pseudomonas* PS-1 (highest HCN producer) and PS-II (higher HCN producer) reduce incidence of collar rot of sunflower by 69.8 per cent and 59.9 per cent respectively, in *Sclerotium rolfsii* infested soil. Our results also revealed that seed bacterization with *P. fluorescens* induce accumulation of higher HCN content and may reduce the incidence of dry root rot of *Jatropha* caused by *R. bataticola*.

Conclusions

From the present study, it could be concluded that seed bacterization with *Pseudomonas fluorescens* induce accumulation of the secondary metabolites viz., [salicylic acid (SA), jasmonic acid (JA), hydrocyanic acid (HCN) and chitinase activity] and may reduce the incidence of dry root rot of Jatropha caused by *Rhizoctonia bataticola*. The higher levels of these metabolites were recorded at 10 days of inoculation in *P. fluorescens* + *R. bataticola* inoculated plants. The secondary metabolites elevated during infection serve as anti-metabolites to pathogen for checking their spread inside the plant through induced systemic resistance (ISR) or systemic acquired resistance (SAR). The pathogen challenged plants showed lower levels of these metabolites.

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