Biological control of coffee leaf rust pathogen, *Hemileia vastatrix* Berkeley and Broome using *Bacillus subtilis* and *Pseudomonas fluorescens*

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ABSTRACT

Bacterial antagonists isolated from coffee rhizosphere soils were evaluated at different concentrations alone and in combination against *Hemileia vastatrix*, the causal organism of leaf rust disease of coffee under *in vitro* and *in vivo* conditions. Under *in vitro* conditions, the rhizobacterium, *Bacillus subtilis* inhibited the growth of urediospores to an extent of 68.20% at a test dosage of 1 x 10^9 cfu ml^{-1} followed by *Pseudomonas fluorescens* to an extent of 64.50% at the same test dose. Combination of *P. fluorescens* and *B. subtilis* at 1 x 10^8 cfu ml^{-1} inhibited the growth of urediospores of the CLR pathogen to an extent of 61.46%. The natural inhibition of germination in the check treatment was 37.79%. Among fungicides used for comparison, the per cent inhibition over control was maximum in Bayleton (89.03%) followed by Bordeaux mixture (80.64%). Under *in vivo* conditions, during the period of season-I, maximum reduction in disease index was recorded in treatment with Bayleton (71.84%) followed by Bordeaux mixture (53.37%). Among the bioagents, *B. subtilis* recorded maximum disease reduction (42.98%) at a test dosage of 1 x 10^9 cfu ml^{-1} followed by *P. fluorescens* (33.65 %) at the same test dose. *P. fluorescens* and *B. subtilis* in combination at 1 x 10^8 cfu ml^{-1} reduced the disease incidence to an extent of 26.45%. The season-II (post-monsoon period) has recorded less disease reduction compared to season-I (pre-monsoon period).

Key words: *Hemileia vastatrix*, arabica coffee, bacterial antagonists.

INTRODUCTION

Coffee trees belong to the genus *Coffea* L. of the family Rubiaceae. *Coffea arabica* L. (arabica coffee) and *Coffea canephora* Pierre ex Froehner (robusta coffee) are the two species of *Coffea* now commercially cultivated throughout the coffee growing countries. Arabica coffee is highly susceptible to many major diseases compared to robusta coffee (Anonymous, 2003). In India, the cultivated area under arabica coffee is about 1.79 lakh ha. with a produ ction of 0.99 lakh MT. The average productivity was 657 kg ha^{-1} clean coffee in the year 2006-07 (Anonymous, 2008).

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* Berkeley and Broome is an obligate parasite and is host specific. It is a major disease of arabica coffee causing economic loss and is reported from over fifty coffee growing countries. The fungus was first observed on coffee in India during 1869 and infects only the foliage and very rarely the young branches. The fungus exists in different physiological forms (races). The uredinial stage is the only viable perpetuating stage of the coffee rust fungus. Wet weather, wind and intermittent rain and sunshine favours the disease development. Urediospores are the infection propagules disseminated by wind, water, insects etc. Severe rust incidence may lead to a foliage loss up to 50% and berries up to 70% (Bhat *et al*., 2000). Hence, effective management of this major disease is of most important in sustained production and productivity of coffee.

At present, adopting cultural practices, planting resistant varieties and application of contact and systemic fungicides are the control measures recommended (Anonymous, 1998). Though satisfactory control of the disease by using various fungicides has been documented (Mayne *et al*., 1933; Muthappa and Nirmala Kumari, 1976; Muthappa, 1981; Daivasikamani and Govindarajan, 1989; Hanumantha *et al*., 1989), continuous use of fungicides may pose many problems like toxicity to non-target organisms, development of resistance in the populations of the pathogen, fungicide residues in the final product and environment and ground water pollution.

In the recent years there is a shift in the control of plant diseases from the regular use of pesticides to an alternate and more eco-friendly biopesticide and plant based products. Many fungi and bacteria have the potential to act as biological control agents. The indigenous strains

of *Bacillus subtilis* and *Pseudomonas fluorescens* appear to function as better antagonists in disease control as they are well adapted to local conditions. Rangeshwaran and Prasad (2000) reported the effectiveness of *P. fluorescens* against chickpea wilt pathogen. Not much work on biocontrol of coffee leaf rust pathogen has been undertaken. The present investigation was carried out to assess the antagonistic effect of *B. subtilis* and *P. fluorescens*, isolated from rhizosphere soils of coffee. The bacterial antagonists were tested *in vitro* and *in vivo* for their effectiveness either in suppressing or controlling the coffee leaf rust pathogen.

MATERIALS AND METHODS

Evaluation of bacterial antagonists under *in vitro* conditions

The bacterial antagonists *B. subtilis* and *P. fluorescens* isolated using standard techniques from coffee rhizosphere soils of Chikmagalur coffee zone were used for the present study. Laboratory studies were carried out at the Central Coffee Research Institute (CCRI), Bale honnur, Chikmagalur, Karnataka during the year 2006. The experiment was laid out in a completely randomized design (CRD). There were ten treatments with five replications. The bacterial antagonists were tested at three different doses (1×10^7, 1×10^8 and 1×10^9 cfu ml^{-1}) individually and in combination (both the bacterium at 1×10^8 cfu ml^{-1}). The recommended fungicides like Bordeaux mixture at 0.5% and Bayleton 25 WP (triadimefon) at 0.02% a.i. were included as treatments for comparison along with a control. Rust infected leaves were collected from the most susceptible arabica coffee genotype (Cauvery) grown at the CCRI farm. The urediospores were collected from urediosori of the infected leaves and ten milligram of urediospores was weighed and added to each of the test dose containing bacterial suspensions and fungicide solutions taken in a 10 ml glass test tube (Corning make). The spores were soaked for 5 minutes in each of the bacterial suspension and fungicide solutions. In sufficient numbers of sterilized Petri plates (9 cm dia.), 15 ml each of 2% water agar medium was poured and allowed to solidify. To each Petri plate, one ml of the fungicide or bacterial suspension containing urediospores were added and uniformly spread over the agar medium. The Petri plates containing only 2% agar inoculated with urediospores of CLR pathogen served as the control. The inoculated Petri plates were incubated over night in darkness at room temperature (24 ± 2°C). After 18 hrs of incubation, the inoculated plates were observed directly under a stereoscopic microscope. The total number of urediospore present in each microscopic field and the number of germinated urediospore were counted. Based on the count, the percentage inhibition over control was estimated.

Field evaluation of bacterial antagonists

Field experiments were conducted for two years during 2006 and 2007 at Gundikhan estate near CCRI. The bacteria tested under *in vitro* conditions were further evaluated in the field for their antagonistic effect against coffee leaf rust (CLR) pathogen. The experiment was laid out in a randomized block design (RBD). Water sprayed plots were considered as control and fungicide sprayed plots were used for comparison. There were ten treatments with a plot size each of 9.0 x 5.4 m^2 with three replications. The plant material used was 15 years old arabica coffee variety Cauvery. The agronomic practices were carried out as per standard recommendations (Anonymous, 2003).

The biocontrol agents, *B. subtilis* and *P. fluorescens* were tested individually at 1×10^7, 1×10^8 and 1×10^9 cfu ml^{-1} concentrations. In combination spray the dose of the agents was kept at 1×10^8 cfu ml^{-1} each. Bordeaux mixture was used at the recommended dose of 0.5% while Bayleton 25 WP was used at 0.02% a.i. Check plants were treated with sterile water. The foliar spray was imposed with high volume ASPEE – Gator sprayer during the last week of May (season I = Pre monsoon) and first week of September (season II = Post monsoon) in the year 2006 and 2007. The adjuvant, Indtron-AE (Indofil Chemicals Company, Mumbai) a non-ionic spreader, sticker and activator was used at 0.3 ml per litre of spray solution. For each treatment, 1500 litres of spray mixture (containing bioagents or fungicides in water) per hectare was used. Observations on percentage of rust incidence in the experimental plot was recorded prior to treatment and thereafter at 15 days interval up to 90 days. In each treatment, fifteen plants were selected for scoring of leaf rust incidence. The per cent disease index was calculated based on the total number of healthy leaves and total number of infected leaves from the tagged branches and the data were tabulated.

Statistical analyses

Data collected from laboratory and field experiments were analysed statistically using standard statistical procedures. The percentage values were subjected to *arc-sine* transformation. The treatment means were compared using least significant difference (LSD) and Duncan's multiple range tests (DMRT) at 5% level of significance (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Bioefficacy under laboratory conditions

The results on *in vitro* bacterial antagonisms of *B. subtilis* and *P. fluorescens* are presented in Table 1. Per cent inhibition of urediospore germination over control in different treatments is presented in the table. The percentage germination of urediospores of *H. vastatrix* in different test doses of the bioagents was compared with the fungicides as well as untreated control. Lowest spore germination (6.82%) was observed in Bayleton fungicide treatment. Among the two bacterial antagonists, *B. subtilis* recorded less spore germination (19.78%) compared to *P. fluorescens* (22.08%) at the higher concentrations (1 x 10^9 cfu ml^{-1}) tested. A maximum percentage of (62.21%) germination of uredospore was observed in control treatment.

Among the fungicides, the maximum per cent inhibition over control was observed in Bayleton (89.03%) followed by Bordeaux mixture (80.64%). The rhizobacterium, *B. subtilis* inhibited the growth of urediospores (68.20%) at a test dosage of 1 x 10^9 cfu ml^{-1} followed by *P. fluorescens* (64.50%) at the same test dose. Combination of *P. fluorescens* and *B. subtilis* at 1 x 10^8 cfu ml^{-1} inhibited the growth of urediospores of the CLR pathogen to an extent of 61.46%. The laboratory studies indicated that the antagonists either *P. fluorescens* or *B. subtilis* could be used alone or in combination for the management of coffee leaf rust disease as a possible alternative to fungicides after appropriate field evaluation.

Field efficacy of biological control agents

Based on the mean values of individual season, the percentage of disease reduction in a season by the bacterial antagonist at different doses and also the treatment effect of fungicides were compared with water sprayed control. The data are presented in Table 2.

The cumulative data on mean per cent disease reduction of two seasons over a period of two years indicated that during season-I (pre-monsoon period), maximum reduction in disease index was recorded in fungicide Bayleton treatment (71.84%) followed by Bordeaux mixture (53.37%). Among the bioagents, *B. subtilis* recorded maximum disease reduction (42.98%) at a test dosage of 1 x 10^9 cfu ml^{-1} followed by *P. fluorescens* (33.65 %) at the same test dose. *P. fluorescens* and *B. subtilis* in combination at 1 x 10^8 cfu ml^{-1} have reduced the disease incidence to an extent of 26.45% in the field. The cumulative disease reduction during the post-monsoon period (season-II) in fungicide Bayleton treatment was 55.29% followed by Bordeaux mixture 35.42%. Among the bioagents, *B. subtilis* recorded maximum disease reduction (28.40%) at a test dosage of 1 x 10^9 cfu ml^{-1} followed by *P. fluorescens* (22.54 %) at the same test dose. *P. fluorescens* and B. *subtilis* combination at 1 x 10^8 cfu ml^{-1} have reduced the disease incidence to an extent of 17.68% under field conditions. Between seasons within a year, the season I (pre-monsoon period) in both the years of experiment recorded considerably higher

Table 1. Effect of bacterial antagonists on germination of urediospores of *H. vastatrix*

Treatments	Germination percentage	`% inhibition over control
T_1 - *P. fluorescens* @ 1 x 10^9 cfu ml^{-1}	22.08 (27.92)^{bc}	64.50 (53.10)^{cd}
T_2 - *P. fluorescens* @ 1 x 10^8 cfu ml^{-1}	25.03 (30.01)^{bc}	59.76 (50.25)^{de}
T_3 - *P. fluorescens* @ 1 x 10^7 cfu ml^{-1}	32.58 (34.37)^{d}	47.62 (43.20)^{f}
T_4 - *B. subtilis* @ 1 x 10^9 cfu ml^{-1}	19.78 (25.81)^{b}	68.20 (55.40)^{c}
T_5 - *B. subtilis* @ 1 x 10^8 cfu ml^{-1}	24.33 (29.07)^{bc}	60.89 (50.81)^{de}
T_6 - *B. subtilis* @ 1 x 10^7 cfu ml^{-1}	27.12 (31.22)^{cd}	56.40 (48.50)^{e}
T_7 - *P. fluorescens* @ 1 x 10^8 cfu ml^{-1} + *B. subtilis* @ 1 x 10^8 cfu ml^{-1}	23.97 (28.76)^{bc}	61.46 (51.00)^{de}
T_8 - Bordeaux mixture @ 0.5%	12.04 (20.17)^{a}	80.64 (63.11)^{b}
T_9 - Bayleton 25 WP @ 0.02% a.i.	6.82 (14.18)^{a}	89.03 (70.63)^{a}
T_{10} - Un-treated control (Sterile distilled water)	62.21 (51.84)^{e}	-
SEM ±	3.12	2.98
CD (P=0.05)	5.85	6.41

Figures in parentheses are *arc-sine* transformed values; In a column, means followed by same letter(s) are not significantly different at P=0.05 as per LSD.

Table 2. Effect of bacterial antagonists on coffee leaf rust pathogen (Cumulative mean of two years and four seasons)

Treatments	Per cent disease reduction over control				Pooled Mean	
	Year 2006		Year 2007			
	Season I	Season II	Season I	Season II	Season I	Season II
T_1 - *P. fluorescens* @ 1 x10^9 cfu ml^{-1}	36.67 (36.85)^{d}	21.06 (27.25)^{cd}	30.63 (33.20)^{c}	24.01 (29.32)^{cd}	33.65 (34.15)^{d}	22.54 (28.19)^{d}
T_2 - *P. fluorescens* @ 1 x 10^8 cfu ml^{-1}	20.53 (26.66)^{ef}	9.74 (17.75)^{ef}	11.35 (19.47)^{ef}	11.41 (19.46)^{ef}	15.94 (22.72)^{f}	10.58 (18.27)^{fg}
T_3 - *P. fluorescens* @ 1 x 10^7 cfu ml^{-1}	10.49 (18.71)^{g}	3.72 (9.87)^{f}	5.31 (12.85)^{f}	6.05 (14.13)^{f}	7.90 (15.12)^{g}	4.89 (10.15)^{h}
T_4 - *B. subtilis* @ 1 x 10^9 cfu ml^{-1}	46.75 (42.67)^{c}	26.85 (30.63)^{bc}	39.21 (38.66)^{c}	29.94 (32.49)^{bc}	42.98 (40.21)^{c}	28.40 (31.72)^{c}
T_5 - *B. subtilis* @ 1 x 10^8 cfu ml^{-1}	22.03 (27.92)^{e}	10.74 (18.39)^{ef}	14.37 (21.96)^{de}	13.91 (21.78)^{ef}	18.20 (25.09)^{f}	12.33 (20.19)^{f}
T_6 - *B. subtilis* @ 1 x 10^7 cfu ml^{-1}	12.26 (20.26)^{fg}	4.82 (11.66)^{f}	7.85 (15.31)^{ef}	8.53 (16.38)^{f}	10.06 (18.17)^{g}	6.68 (14.22)^{gh}
T_7 - *P. fluorescens* + *B. subtilis* @ 1 x 10^8 + 1 x 10^8 cfu ml^{-1}	31.46 (33.86)^{d}	16.42 (23.55)^{de}	21.44 (28.27)^{d}	18.93 (25.12)^{de}	26.45 (29.53)^{e}	17.68 (24.12)^{e}
T_8 - Bayleton 25 WP @ 0.02% a.i.	76.81 (60.63)^{a}	55.79 (47.86)^{a}	66.87 (54.33)^{a}	54.78 (47.25)^{a}	71.84 (58.32)^{a}	55.29 (46.39)^{a}
T_9 - Bordeaux mixture @ 0.5%	56.51 (48.43)^{b}	34.34 (35.63)^{b}	50.22 (45.03)^{b}	36.49 (36.83)^{b}	53.37 (46.20)^{b}	35.42 (36.09)^{b}

Figures in parentheses are *arc-sine* transformed values; In a column, means followed by same letter(s) are not significantly different at P=0.05 as per DMRT.

percentage of disease reduction over season II (post-monsoon period). This could be due to the reason of higher levels of rust incidence generally recorded in the plantation during the post-monsoon period (season II).

Both under *in vitro* and *in vivo* experiments, with the use of bacterial antagonists against the coffee rust pathogen indicated that the coffee rust disease incidence could be brought down considerably without disturbing the equilibrium of the environment if applied at appropriate time. This indigenous, environment friendly and non-pollutant bacterial antagonists have to be further exploited in the biocontrol approach for better management of coffee leaf rust pathogen.

This constitutes the first report from India on the *in vitro* and *in vivo* testing of bacterial antagonists as a tool for biological control of coffee leaf rust disease.

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