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Management of Chilli Anthracnose by the Biopreservative Effect of Lactic Acid Bacteria

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ABSTRACT

The biopreservation of fruits and vegetables using lactic acid bacteria is an innovative approach that helps in long term storage and improvement of the quality of the produce by minimizing the effect of storage fungi on perishables. In this study, Lactic Acid Bacterial (LAB) isolates were screened against Colletotrichum capsici Syd., causing anthracnose in chilli. The isolates LAB 75 and LAB 82 were more promising in inhibiting the growth of the fungus. In the in vivo assays, LAB 75 was more effective in reducing the severity of anthracnose disease on chilli fruits, under challenge inoculated conditions. Spraying with LAB 75 on to chilli fruits resulted in a lower per cent disease index (25.13 PDI) when compared to 59.36 PDI in the pathogen control after six days of challenge inoculation, accounting to about 60 per cent decrease in PDI. Further, the LAB population was the highest on chilli fruits in this treatment (300x104 cfu1 g). Thus, LAB 75 proved its potential to be used as a bio-preservative of chilli fruits by effectively managing anthracnose of chilli.

KEYWORDS : Anthracnose, Chilli, lactic acid bacteria, Colletotrichum capsici , biopreservative

Introduction

Chilli (*Capsicum annuum* L.) is one of the most popular and widely grown vegetables in India (Makari *et al*., 2009). It is grown in 8.26 lakh ha with an yield of 5.11 lakh tones of dry chili annually (Anon., 2010). Andhra Pradesh and Karnataka account for 75 per cent of the country's net area under chili. India is the largest exporter of chili and about 2.5 to 3.0% of country's total production is exported. However, anthracnose disease caused by *Colletotrichum capsici* Syd.is the most economically important constraint which is hampering production in major chilli growing regions of the country, accounting to 12-25 per cent yield losses (Sharma *et al*., 2005). The disease is characterized by the production of symptoms on leaves, stem and fruits and causes severe damage to mature fruits in the field. Moreover, during transit and storage, this disease also causes severe damage to chilli fruit (Mehrotra and Aggarwal, 2003). Anthracnose infection on fruits causes blemishes and fruits become unmarketable. The typical symptoms on chilli fruits are characterized by the formation of sunken necrotic lesion, with concentric rings of acervuli.

Although chemical pesticides can be used to manage the anthracnose disease, they are uneconomical and health hazardous as the fruits are directly consumed. Lactic acid bacteria (LAB) are generally recognized as safe (GRAS microorganisms) and play a pivotal role in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) which result in lowered pH (Daeschel, 1989). LAB also produce antimicrobial compounds including hydrogen peroxide, CO2, diacetyl, acetaldehyde, isomers of amino acids, reuterin and bacteriocins (Cintas *et al*. 2001). An additional advantage of the LAB strains is the absence of adverse effects of spoilage, such as browning or off odors. The survival of LAB in post harvest conditions makes these bacteria most efficient to prevent post harvest spoilage.

Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products (Ibourahema *et al*. 2008), in the present study, we screened a large collection of lactic acid bacteria, isolated from diverse sources, maintained in the Department of Agricultural Microbiology, UAS, Dharwad, for their antagonistic effects *in vitro* against *C. annuum* and selected the promising ones and evaluated them on fresh cut fruits of chilli after challenge inoculation with *C. annuum*.

Materials and Methods

Isolation and purification of *Colletotrichum capsici* from the diseased chilli fruits: Chilli fruits (*Capsicum annum*) infected with anthracnose were collected from field and used for isolation of the fungus *in vitro*. The isolation of the fungus was made by following the standard tissue isolation technique. Colonies, which developed from these bits, were identified by microscopic observation by taking mycelial and spore characters as means for identifying the pathogen (Cannon *et al.,* 2000). The morphological characters of the fungus such as acervuli, setae and conidia were studied under a microscope. The morphological characters were compared with the characters of *Colletotrichum capsici.*

Conducting the pathogenicity test on chilli fruits and confirmation of the pathogen: For inoculation, healthy chilli fruits were used. The fruits were inoculated by following pin pricking and spray method. *Colletotrichum capsici* spores (2x104 spores/ml) were taken, with the help of sterilized needle, from one month old culture and this needle was pricked into the pericarp of the healthy chilli fruits. The pathogen was reisolated from the diseased fruits and the morphological characters of *C. capsici such as* acervulus, setae and conidia were studied.

In vitro screening of LAB isolates for antagonistic activity against C. capsici: *In vitro* evaluation of 41 LAB isolates was carried out following the dual culture method, in which 1ml of spore suspension of the pathogen (*C. capsici*) was spread over a petri plate containing PDA, in order to have a lawn of the pathogen. After drying, 100 µl of freshly prepared LAB cultures were spot inoculated on the lawn and the plates incubated at 30^0 C for 7 days. Further, the observations were made for the zone of inhibition.

In vivo evaluation of the isolates for enhancing the shelf life of chilli fruits and identification of the suitable delivery method: One kg fresh chilli fruits were used for each treatment. The selected LAB isolates (75 and 82) were grown in MRS liquid medium for two days and the population estimated. The growth was diluted with sterile water (1;3) (Yang *et al*., 2012). The chilli fruits were given LAB treatments as per the treatment schedule. *C. capsici* was grown on several PDA plates for 10 days and the spores harvested and collected in sterile water. Using a haemocytometer, the spore population was adjusted to 10^4 per ml (Yang *et al*., 2012) and sprayed on these fruits after three days of LAB inoculation. The fruits were air dried and incubated in the form of heaps in a glass house at 30^0 C. The experiment was monitored for the disease expression and the disease

scored at regular intervals of time.

Table 1:Treatment schedule:

Treatments	Treatment details
T_1	Absolute check (No LAB, No pathogen i.e. healthy check)
T_2	Pathogen check (only pathogen)
T_3	Pathogen + LAB 75 spraying
T_4	Pathogen + LAB $_{75}$ dipping (30 min.)
T_5	Pathogen + LAB $_{75}$ spraying and dipping
T_6	Pathogen + LAB $_{82}$ spraying
T_7	Pathogen + LAB $_{82}$ dipping (30 min.)
T_8	Pathogen + LAB $_{82}$ spraying and dipping
T_9	Pathogen + LAB75 and LAB $_{82}$ (1:1) spraying
T_{10}	Pathogen + LAB75 and LAB $_{82}$ (1:1) dipping (30 min.)
T_{11}	Pathogen + LAB75 and LAB $_{82}$ (1:1) spraying and dipping
T_{12}	Pathogen + Chemical spraying (0.1%)

Disease grading: Anthracnose of chilli incidence was recorded by scoring 50 chilli fruits in each treatment using 0-9 scale (Mayee and Datar, 1986) as under.

Table 2: Disease scoring

Grade	Per cent fruit infection
0	No infection
1	1-10
3	11-25
5	26-50
7	51-75
9	>75

Results and Discussion

The results on use of LAB as biopreservative in managing anthracnose of chilli are presented in Tables 3 to 5. Spoilage of vegetables by moulds and yeasts can be reduced or delayed by good sanitation practices, careful handling during harvesting, proper transportation and storage conditions (temperature and humidity) and use of chemical preservatives., In recent years, increasing interest has been shown in biopreservation i.e., the use of microorganisms or their metabolites (Davidson 2001) over chemical preservation due to consumer demand for a reduced use of chemicals in food and feed because of global food safety measures and to reduce health hazards, such as indigestibility or allergies (Ross *et al*. 2002; Devlieghere *et al*. 2004). In this investigation, the pathogen, *C. capsici* was isolated from the Chilli fruits infected with anthracnose, purified and the pathogenicity confirmed. Based on the typical symptoms of the disease as well as the morphological characters of the colonies and the microscopic observations of the acervuli, setae and conidia, the fungus was identified as *C. capsici.* On the fruits, typical small circular spots appeared, enlarged in the advance stage, became more or less elliptical and caused fruit rot. These spots expanded in the direction of long axis on the fruit and became black or dark brown in colour. In these lesions, black pinheads like fruiting bodies were found in concentric rings. The conidia were one- celled, smooth walled, hyaline, falcate with sometime fusiform, tapered towards both ends and acute at the apex (Shenoy *et al*., 2007).

Application of lactic acid bacteria (LAB) in traditional food and feed fermentation and preservation has been well documented (Kim 1993). Their potential application for prevention of vegetable spoilage opens up a new area in the biopreservation technology. Hence, large collections of the LAB were screened for inhibition of *C. capsici*. It was observed that very few strains showed the zone of inhibition activity. Out of 43 isolates, only two resulted in zone of inhibition as high as 19 mm and 17 mm by LAB 75 and LAB 82 isolates respectively. Hence, these strains were selected for the subsequent *in vivo* biopreservation studies. Similarly, Heng *et al*., (2011) also observed the zone of inhibition of *C. capsici* produced by actinobacterial isolates in the range from 6 to 12 mm. As many as seven LAB isolates showed good inhibition activity against *C. gloeosporioides* by dual overlay method and well method (El-Mabrok *et al*., 2013). The broad spectrum antifungal activities of lactic acid bacteria have also been reported against *Aspergillus fumigatus, A. nidulans, Penicillium commune* and *Fusarium sporotrichioide (*Magnusson *et al*., 2003). The cell free extract of LAB inhibited growth of *Penicillium expansum, Botryti cinerea* and *Monilinia fructicola* (Yang *et al*., 2012).

In the biopreservation studies, the chilli fruits were given treatments with the LAB strains in terms of spraying, dipping and both.The extent of anthracnose disease severity was assessed at different periods of incubation. It is evident from Tables 3, 4 and 5 that LAB 75 was more effective in reducing the severity of anthracnose on chilli fruits, under challenge inoculated conditions. The Per cent Disease Index (PDI) was significantly reduced from 59.36 per cent (in control) to 25.13 per cent by spraying with LAB 75 strain after six days of challenge inoculation (DAI). That means, the PDI was reduced by about 60 per cent. This trend continued till 14 DAI. The number of healthy fruits was also higher in the LAB 75 sprayed chilli treatment. (6.18%) when compared to the pathogen control (3.85%) after 18 DAI. In the identification of the most suitable delivery method, spraying was found superior when compared to dipping in LAB for 30 min.

It has been hypothesized that organic acids act on the cytoplasmic membrane by neutralizing its electrochemical potential and increasing its permeability, thus leading to death of the organism (Dalie *et al*., 2010). Magnusson and Schnurer (2001) found that the antifungal metabolite produced by *Lacobacillus coryniformis* subsp. *coryniformis* strain Si3 was a small highly heat stable peptide and its activity was stable at pH between 3.0 and 4.5, but rapidly decreased between 4.5 and 6.0 and no inhibitory activity being observed at above pH 6.0. Yang *et al*., (2012) observed biopreservative effect of LAB against *Pseudomonas* and *Enterobacter* which were inoculated on to fresh cut onions. *Listeria innocua* inoculated on to fresh-cut onions was reduced by 1 to 1.6 log cfu g^{-1} after 12 days storage at 5^0 C due to the presence of selected LAB strains. Similarly, Sharpe (2009) observed that *L. lactis* and *Enterobacter faecium,* when inoculated on to fresh-cut salads, inhibited the growth of *Pseudomonas*, yeast and total coliforms, thus bringing a biopreservative effect.

In the present study, when assayed for the population of LAB on chilli fruits, it was found to be on a higher side ($300x10^4$ cfu g^{-1}) when compared to other treatments. Thus, LAB 75 exhibited a biopreservative effect on chilli fruits against the anthracnose disease caused by *C. capsici*. Further investigation is needed to establish the mode of action of the LAB isolate.

Conclusion

LAB 75 strain was found to be more efficient in the *in-vitro* inhibition of *Colletotrichum capsici* and also in the *in vivo* assays. It effectively reduced the disease severity of anthracnose and protected chilli fruits from deterioration even after 10 days of infection and found on par with the chemical preservation up to 17 days. LAB 75 strain could be used as an ideal bio-preservative of chilli fruits for long term storage without affecting quality of the produce. Thus, the present findings opened a new window of opportunity for managing anthracnose of chilli in India.

Table 3: Effect of LAB treatments on the PDI of anthracnose on chili fruits on different days (under challenge conditions) (Values in brackets represent arc sine transformed values)

Treatments	PDI of anthracnose on chili fruits on different days					
	3 DAI	6 DAI	8 DAI	10 DAI	12 DAI	14 DAI
T_1	13.88 (21.6)	24.05(29.3)	32.00(34.4)	37.81(37.9)	43.58(41.2)	45.35(42.2)
T_2	47.35 (43.5)	59.36(50.4)	67.37(55.1)	68.03(55.6)	70.27(56.9)	73.50(59.0)
T_3	20.00 (26.4)	25.13(30.1)	49.13(44.4)	52.92(46.6)	56.49(48.7)	58.92(50.1)
T_4	44.25 (41.6)	47.81(43.7)	50.02(44.9)	52.49(46.4)	54.49(47.5)	59.37(50.3)
T_5	36.69(37.0)	43.14(41.0)	50.69(45.4)	55.59(48.2)	59.15(50.2)	61.36(51.5)
T_6	36.02(36.8)	50.25(45.1)	55.59(48.2)	58.26(49.7)	61.36(51.5)	63.60(52.8)
T_7	31.58(34.2)	37.81(37.9)	42.24(40.5)	50.25(45.1)	55.59(48.2)	59.15(50.2)
T_8	31.35(34.0)	54.92(47.8)	57.82(49.4)	59.59(50.5)	66.26(54.5)	69.37(56.3)
T_9	46.26(42.8)	52.49(46.4)	63.15(52.5)	67.16(55.0)	68.93(56.1)	70.04(56.8)
T_{10}	46.93(43.2)	58.04(49.6)	63.00(52.6)	64.49(53.4)	66.05(54.3)	70.70(57.2)
T_{11}	43.80(41.4)	46.48(42.9)	53.81(47.0)	61.36(51.5)	63.60(52.8)	67.37(55.1)
T_{12}	29.80(33.0)	32.00(34.4)	35.57(36.6)	41.35(40.0)	44.91(42.0)	47.15(43.3)
CD @ 5%	1.261	1.323	1.200	1.929	2.145	1.699
SEm	0.430	0.451	0.409	0.658	0.731	0.579

Table 4: Effect of LAB treatments on the incidence of anthracnose in chili fruits (after 18 DAI).

Treatments	Total chilli fruits in a heap	No. of Healthy fruits	No. of diseased fruits	% healthy fruits
T_1	580	66	514	11.37
T_2	648	25	623	3.85
T_3	631	39	592	6.18
T_4	629	31	590	4.92
T_5	605	40	565	6.61
T_6	637	42	595	6.59
T_7	636	34	602	5.34
T_8	630	33	597	5.23
T_9	630	28	602	4.44
T_{10}	611	23	588	3.76
T_{11}	602	29	573	4.81
T_{12}	576	63	513	10.93
CD @ 5%	-	4.943	NS	-
SEm	-	1.685	9.838	-

Table 5: Population of LAB on chilli fruits as influenced by PGPR treatment

Treatment	LAB population ($CFUx10^4/g$)
T_1	000
T_2	000
T_3	300
T_4	253
T_5	296
T_6	067
T_7	034
T_8	219
T_9	285
T_{10}	075
T_{11}	284
T_{12}	010

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