Original Research Article

The biocontrol of soil transmitted *Cercospora capsici* with *Lactobacillus plantarum*

ABSTRACT

Microbial diseases of pepper (*Capsicum species*) are the most significant factors contributing to the loss of this economically important vegetable crop. Leaf spot disease caused by *Cercospora capsici* is one of the major constraints to pepper production in Nigeria. The disease is reportedly introduced through infected seeds. However, the development of Cercospora leaf spot on plants propagated from fungicide-treated seeds suggests an alternative perennial source of infection and also necessitates the development of an efficient, safe control measure. *C. capsici* was isolated from infected pepper plant through the direct plating method and subsequently characterised. Treated pepper seeds (with *L. plantarum*) were planted in *C. capsici* inoculated soil, while the emergence, seedling growth parameters and severity of leaf spot were observed. The severity index of Cercospora leaf spot on pepper plants (observed at the 20th day after planting) was significantly lower on *L. plantarum* treated plant set (0.07) than on pepper without seed treatment. Seed emergence rate index increased from 11.11 to 15.33 %/day of untreated to treated pepper seeds sown in infected soil respectively, while the mean emergence time of untreated seeds (8.32 days) was significantly higher in *C. capsici* infected soil. It could therefore be deduced that seed priming with *L. plantarum* improved the seedling vigor and resistance of pepper to leaf spot disease caused by *C. capsici.*

Keywords: Pepper, Cercospora capsici, Lactobacillus plantarum, biocontrol

1. INTRODUCTION

Capsicum chinense Jacq. belongs to the family Solanaceae, it is one of the important cash earning crops globally, mainly cultivated as a fruit vegetable [1]. It is a commercially important vegetable crop in Nigeria [2], grown in almost all the States under rainfed or irrigated conditions for both domestic and export (through the Nigerian Export Promotion Council) purposes. Nigeria accounts for about 50% of African pepper production, making Nigeria the largest producer of pepper in tropical Africa [3]. Chilli pepper has been described as an increasingly important vegetable crop. It is used for culinary purposes as spice and food colorant; as well as for medicinal applications. The consumption of Chilli pepper has, over the last 35 years, increased beyond 40-folds [4]. This economically significant vegetable crop is an important source of essential food nutrients, including vitamins K, C, B and lycopene (carotenoid) calcium and trace metals, which could provide a long term health benefit to consumers [5]. Natural, bioactive, pharmacological compounds associated with pepper includes the phytochemical, capsaicin, essential oils, a rich combination of organic micronutrients and Reactive Oxygen Species (ROS) protective antioxidants [6].

The production of pepper in Nigeria is limited by several factors including diseases caused by fungi, bacteria, viruses and nematodes, as well as physiological imbalances caused by abiotic stress. Among the microbial diseases responsible for pepper yield loss worldwide, fungal diseases are the most frequent [1]. Leaf spot caused by *Cercospora capsici* Heald and Wolf is a major constraint to pepper production in Nigeria, causing foliar pathogen damage with epidemic potentials, due to its exponential spread among

susceptible varieties [7]. Cercospora leaf spot usually begins to manifest at the base or lower surface of infected leaves, where there is more moisture and less wind or air circulation and subsequently spreads outwards. The symptoms on leaves appeared as circular (up to 1 cm diameter), small, brown to black spots with a small (0.5–1 mm) whitish centre that slowly enlarges, which could become severely necrotic and sometimes accompanied with leaf chlorosis. Although, *C. capsici* is mostly introduced through infected pepper seeds, the emergence of Cercospora leaf spots on pepper plants grown from treated seeds suggests other sources of primary infection. It is therefore imperative to investigate the potentials of soil as a reservoir of inoculum for pepper, considering the possibilities of infected crop residues (within the soil) as a platform of seasonal pathogen carryover.

The use of chemical fungicides to control microbial diseases of plants leads to the accumulation of toxic chemicals, which could pose a potential threat to human health upon consumption and their non-selective toxicity could negatively alter the balance of soil beneficial microorganisms [8]. However, Lactic Acid Bacteria (LAB), especially, *Lactobacillus plantarum,* had been documented to inhibit several animal and plant pathogens *in vivo* and *in vitro* without generating undesirable effects on the host [9]. The alternative use of these antagonistic Generally Regarded as Safe (GRAS) microorganisms to control phytopathogens of pepper would improve the survival of pepper, reduce the yield loss currently associated with this vegetable crop and improve its production in Nigeria. Consequently, this research is designed to investigate the control effect of *Lactobacillus plantarum* on Cercospora leaf spot disease of pepper.

2. MATERIALS AND METHODS

2.1 Sample collection and experimental sites

Infected plant samples showing symptoms of Cercospora leaf spots were collected from different farms at the Ibadan/Ibarapa agricultural zone, Oyo State, Nigeria (7°23'39.6"N; 3°47'02.9"E /7°26'14.5"N; 3°16'3.4"E). Samples were collected in bags, transported to the laboratory and kept in the refrigerator at 4 °C. Laboratory analyses were carried out at the Microbiology Laboratory of the Federal College of Animal Health and Production, while the pot experiment was done at the Horticulture unit, Federal College of Agriculture, Moor Plantation, Ibadan (7°22'48.5"N; 3°50'29.0"E).

2.2 Isolation of *C. capsici* from infected pepper plants

Using a sterile scalpel, 5 mm portion of infected pepper leaf was cut from each plant sample. The separated plant tissues were surfaced sterilized with 1 % sodium hypochlorite (NaOCl) solution for 2 minutes. Treated samples were subsequently rinsed thrice in sterile distilled water (SDW), drained on sterile filter paper and inoculated on solidified, presterilised (121 °C at 15 psi for 15 minutes) Potato Dextrose Agar (PDA) using the direct plating method. Three replicates were made for each sample and the inoculated Petri plates were incubated at ambient temperature (25±2) for 5-7 days. Fungal growth on each plate was then subcultured on fresh PDA plates to obtain pure cultures of each strain [2].

2.3 Identification of isolated fungi

The purified fungal isolates were identified by examining them macroscopically and microscopically. Colony properties such as the appearance, change in medium colour and growth rate were observed. Microscopic structures like the shape of hyphae, conidia and conidiophores were observed. These structural features were compared with standards [10].

2.4 Pathogenicity test

Pathogenicity test was carried out to investigate the aggressiveness and measure the virulence of each infected plant. Fungal suspension was prepared for soil inoculation. This was done by subculturing (on PDA) pure isolates from the edge of an actively growing plate and incubating at room temperature for 5 days. Sterile distilled water (30 mL) was thereafter poured over the surface of the fungal growth and a sterile spatula was used to scrub the surface of the fungus to dislodge the mycelium over a sterile cheesecloth filter

to collect the fungal spores (filtrate). Hemocytometer was used to adjust the spore concentration of the fungal suspension to 10^5 conidia/mL. Presterilized sandy loam soil (5 kg/pot) was then inoculated with 25 mL of the prepared fungal suspension a day prior to planting and the pot was kept humid with SDW [11]. Seeds of *Capsicum chinense* Jacq., cv. Rodo, a variety commonly cultivated due to its early emergence, were surface sterilised with 1 % NaOCl for two minutes, drained on sterile filter paper and planted 24 hours after soil inoculation. The manifestation leaf spots was recorded and compared. The most virulent strain was re-characterised (identified) and kept in the refrigerator at 4 °C for subsequent analyses.

2.5 *In vivo* control of Cercospora leaf spot with *L. plantarum*

2.5.1 Seed treatment

Soil inoculation was done as earlier described in section 3.4 and seed treatment with LAB species was carried out by biopriming surface sterilised seeds with 10^6 CFU/mL purified *L. plantarum* (Earth's pearl™) and planted in inoculated soil (15 mm depth) at the rate of 8 seeds per pot [12]. Negative control seeds and soil were treated with SDW (treatment-C), while the soil of treatment-A plant set was inoculated with *C. capsici* without seed treatment. Both the soil and the seeds of treatment-B plants were inoculated with the pathogen and *Lactobacillus* species respectively. The seeds of treatment D plant set were bioprimed with *L. plantarum* without soil treatment. Emergence was scored when the hypocotyl hook was seen on the soil surface, while emergence parameters were measured and seedling properties were recorded for 25 days after planting.

2.5.2 Measurement of control and biostimulating effects of *L. plantarum*

The severity scale of 1-5, as described by Asare-Bediako *et al.* [13], was used to investigate the control effect of seed treatment and the scoring was translated to disease severity index (DSI).

$$\%\ \text{Disease severity index (DSI)} = \frac{\sum(\text{Severity score} \times \text{Number of plants})}{\text{Highest severity score} \times \text{total number of plants}} \times 100$$

[14]

Seeding dry weight (SDW) and seeding fresh weight (SFW) were determined, while Emergence percentage, emergence rate index [15], seed vigor index, mean emergence time [16] and disease incidence [11] were measured as described below:

I. **Emergence percentage**

$$\text{Emergence (\%)} = \frac{\text{Number of emerged seeds}}{\text{Total number of seeds}} \times 100$$

II. **Emergence rate index (ERI)**

$$\text{Emergence rate index (\%/day)} = \frac{G5\%}{5} + \frac{G10\%}{10} + \frac{G15\%}{15} + \cdots \frac{G35\%}{35}$$

Where Gx% is the percentage emergence at day x after planting

III. **Seed vigor index (SVI)**

$$\text{Seed vigor index} = \text{Seedling length} \times \text{G35\%}$$

Where G35% is the percentage emergence at day 35 after planting

IV. **Mean emergence time (MET)**

$$\text{Mean emergence time (day)} = \frac{\sum(\text{Emergence Number} \times \text{day})}{\sum(\text{Emergence Number})}$$

V. **Disease incidence**

$$\%\ \text{Incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

2.6 Statistical analysis

The recorded data were subjected to one-way analysis of variance (ANOVA) with the aid of Statistical Package for the Social Sciences (SPSS) software adapted for Windows version 16.0. Each treatment was replicated four times in a Completely Randomized Design (CRD). Analysed means were separated with the Duncan Multiple Range Test (DMRT at P≤0.05).

3. RESULTS AND DISCUSSION

3.1 Identification of *C. capsici*

The isolate was observed to form a light brown mycelial growth upon macroscopic morphological examination. The growth was rapid and irregular, with patches (2-3 cm) of mycelia forming 3-4 days after inoculation. Microscopic examination revealed acicular conidia (55-140 x 3-5 µm), hyaline hypha, septate (5-8) and smooth. The hyphae are either curved or straight with round tips and basal truncated cells with characteristic scars. The macroscopic, microscopic and disease characteristics were compared to standards and the fungal isolate was identified as *C. capsici.*

3.2 Pathogenicity test

The development and spread of leaf spots by *C. capsici* from the lower to upper surface of the leaf (Figure 1), as well as from the older pepper leaves to the younger ones could probably be due to the amount or types of nutrients, structures and moisture content present on these leaves. As the disease progresses, infected plants that do not collapse or die off from infection have been reported to produce lower fruit yield [7]. Leaf spots, spreading from the lower surface to the upper surface of the leaves, appeared as reddish brown spots which later turned to grey spots. Infected areas formed circular necrotic lesions, most times accompanied with chlorosis, which eventually led to wilting. Aggressiveness of *C. capsici* has been attributed to the ability of most pathogenic strains to produce cercosporin, a phytotoxic polyketide metabolite. The ability to produce cercosporin, as well as other virulence metabolites like enzymes and host specific toxins could significantly enhance virulence in *C. capsici* and aid the manifestation of spots through tissue disruption and depolymerization [17, 18].

Figure 1: Pathogenesis of Cercospora leaf spot. A: Development of spots at the lower surface of leaf. B: Spread of spots to the upper leaf surfaces. C: Wilting and stem collapse

3.3 *In vivo* biocontrol of *C. capsici*

As reported by Islam *et al.* [19] in their investigation on the diagnosis and prescription for Cercospora leaf spot of chilli (using the plant disease diagnosis model) reported various strategies in the control of this disease. They observed significant increment in the severity of untreated pepper plants at 80 and 95 days after planting. This affected the growth parameters, weight per fruit and the number of fruits per plants. The incidence of Cercospora leaf spot (as observed in this study) was reduced by 7.34, 1.14, 0.5 and 1 % at the 5th, 10th, 15th and 20th days after planting respectively (Table 1) through pre-planting seed biotreatment with cell suspension of *L. plantarum*. The disease severity index of Cercospora leaf spot was significantly reduced in *L. plantarum* treated plants, compared to plants grown in infected soil without seed treatment. Highest reduction in the disease severity index (1.0) was observed at the 20th day after planting.

Similar biocontrol results were observed by Devappa and Thejakumar [1], who treated *C. capsici* infected pepper plants with lyophilized culture of *Pseudomonas fluorescens* as an *in vitro* control measure. Disease index (DI) from their study was recorded at 45, 60 and 75 days after transplanting; they reported a percentage DI range of 8.00 to 21.33 for treated plants, as compared to the control plants (22.33 - 45.33 %). Abdel-Aziz *et al.* [20] also recorded a significant reduction in the severity of fungal diseases on plants grown from seeds bioprimed with Lactic Acid Bacterial species. They demonstrated the capability of LAB species as effective biocontrol agent against phytopathogenic fungi through *in vitro* and *in vivo* assays. Strains of *L. plantarum* have been described to produce a complex combination of antimicrobial compounds. These compounds include antimicrobial proteins (bacteriocins), diacetyl, hydrogen peroxide and organic acids with broad to narrow spectrum of inhibitory activities [21]. Comparative genomic analysis of *Lactobacillus plantarum* had been used to investigate over 1425 protein-coding genes (within the core genome of this species), some of which are involved in antibiosis [22]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of partially purified antimicrobial protein from *L. plantarum* revealed a molecular weight range between 12 and 45 kDa [21].

3.4 Effect of seed treatment on the growth of pepper seedlings

Seed treatment with *L. plantarum* also improved the emergence of pepper seeds used in this study (Table 1). Seeds planted in infected soil with LAB treatment had the lowest % emergence (33.33) at the 5th day after planting. Bioprimed seeds planted in uninfected soil had the highest number of leaves at 10, 15 and 20 days after planting. However, biopriming pepper seeds did not significantly influence the seedling height of the plant. Taking cognizance of the primed and unprimed seeds planted in uninfected soil, the improvement in performance of treated pepper seedlings observed in this study could be as a result of direct production of plant growth stimulating metabolites by *L. plantarum*, in addition to direct inhibition or

the competitive exclusion of leaf spot pathogen (*C. capsici*). Samarah *et al.* [15] equally described the growth promoting and protective properties of glucosamine polymers on treated pepper seeds. Nanochitin and chitosan are natural biopolymers of arthropod or microbial origin with biostimulating potentials (including improved seedling growth and enhanced seed germination properties). These long-chain polysaccharide structures could also induce host defense mechanisms through tissue fortification (lignification), expression of enzymes, production of reactive oxygen species and activation of primary, as well as systemic defense mechanisms.

Table 1: Protective and biostimulating effects of *L. plantarum* on pepper

Days after planting (DAP)	Treatment	%Emergence	Seedling height	Leaf number	Incidence (%)	Disease severity index
5 DAP	A	33.33b	3.37	2.33ab	20.17a	0.11a
	B	63.33a	3.92	2.83ab	12.83a	0.04b
	C	56.67a	3.40	2.03b	0.00b	0.00b
	D	60.00a	4.85	3.10a	0.00b	0.00b
	Sig.	*	ns	*	*	*
10 DAP	A	40.00a	4.27	4.03b	2.97a	0.14a
	B	20.00b	4.34	4.40ab	1.83ab	0.06b
	C	23.33ab	5.08	4.67ab	0.00b	0.00c
	D	20.00b	5.87	5.10a	0.00b	0.00c
	Sig.	*	ns	*	*	*
15 DAP	A	6.67	6.06	5.37ab	2.50a	0.16a
	B	10.00	6.67	5.30ab	2.00a	0.07b
	C	6.67	7.20	5.09b	0.00b	0.00c
	D	10.00	7.57	5.97a	0.00b	0.00c
	Sig.	ns	ns	*	*	*
20 DAP	A	0.00	6.18	5.57	2.50a	0.17a
	B	0.00	6.80	6.00	1.50b	0.07b
	C	0.00	7.38	6.17	0.00c	0.00c
	D	0.00	7.83	6.13	0.00c	0.00c
	Sig.	ns	ns	ns	*	*

*ns: not significant; *: Significant*

Mean values with similar letter (s) along the column are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT)

3.5 Effect of seed treatment on the emergence parameters of pepper seedlings

Seed treatment appeared to improve the rate of emergence (emergence rate index), the average emergence period (mean emergence time), seedling dry weight and the seedling vigor index of pepper (Table 2). Infected pepper seedling without *L. plantarum* treatment had the lowest seedling dry weight (0.07 g), mean emergence time (8.32 days), emergence rate index (11.11 %/day) and seedling vigor index (860.00). *L. plantarum* seed treatment significantly biostimulated pepper seeds with a seed vigor index value of 1.350×10^3. In a similar study, the biopriming of wheat seeds with cell suspensions of *L. plantarum* ONU12, *L. plantarum* ONU311 and *L. plantarum* ONU355 strains was reported to increase seed germination by 6.0 to 40.0 % depending on the substrate used and the inoculum concentration. Limanska *et al*. [23] reported an optimum inoculum concentration range of 10^3 to 10^6 CFU/mL for wheat

seed biotreatment under hydroponics and soil conditions. *L. plantarum* was observed to form biofilms on roots of seedlings and improved the height, as well the root length of host plants [23].

Similar biostimulating results have been reported on other nightshade crops. Tomato seeds primed with *Streptomyces fradiae* NKZ-259 suspension improved seed emergence and seedling vigor through the production of Indole-3-acetic acid [24]. Mavi [25] also evaluated the effect of organic priming on the emergence performance of five domesticated *Capsicum* species (*C. frutescens, C. baccatum*, *C. annuum, C. chinense* and *C. pubescens*). The author reported improved emergence vigor index, higher seedling length values, as well as higher fresh and dry seedling weights of *Capsicum* species primed with aqueous extract of *Tagetes patula* L.

Table 2: Effect of seed treatment with *L. plantarum* on the emergence and performance of pepper

Treatment	SFW (g)	SDW (g)	MET (day)	ERI	SVI ($\times 10^3$)
A	1.37b	0.07b	8.32a	11.11b	0.860c
B	2.13ab	0.20a	7.17b	15.33a	1.161b
C	2.23ab	0.23a	7.10b	13.60a	1.184b
D	2.33a	0.27a	7.21b	15.17a	1.350a

Mean values with similar letter (s) along the column are not significantly different at 5 % level of probability by Duncan Multiple Range Test (DMRT)

4. CONCLUSION

L. plantarum, used as seed treatment, in this study improved the performance of pepper seedling through the suppression of Cercospora leaf spot disease, as well as by direct biostimulation of seedling emergence and growth. However, in addition to its well documented antimicrobial properties, further investigation is imperative to establish the specific pepper growth promoting metabolites and the whole-cell biostimulating mechanisms of this species. This would be important to optimise the potentials of *L. plantarum* and possibly broaden its spectrum of application.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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