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In vitro comparative study of six commercial formulates on bacterial black spot of mango in Kolar and Chitradurga district, Karnataka

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ABSTRACT: *Xanthomonas campestris pv. mangiferaeindicae (Xcm) caused bacterial black spot of mango in Karnataka. The extent to which bactericides control this disease effectively is low. In this study the bactericidal effect of different products was assessed in vitro on mango plants under greenhouse conditions. Six commercial formulate and combinations were tested. In vitro analysis showed that minimal inhibitory concentration (MIC) of copper sulphate with a MIC value of 100 µl/ml and bavistin which was not active at 500 µl/ml. MIC values of commercial formulate bactrinashak ranged between 5 and 30 µg/ml, and combinations of copper oxychloride + copper sulphate; streptocycline + bactrinashak; mancozeb + copper oxychloride, mancozeb + bavistin and bavistin + bactrinashak, showed a great effect at sub-inhibitory concentrations. Treatments including copper sulphate and copper oxychloride significantly shows inhibition zone, whereas bavistin alone was less effective, these combinations of different antibacterial substances results were better than copper sulphate alone. We conclude that the combination of copper sulphate with streptocycline, bactrinashak may be useful in controlling symptoms of this disease in greenhouses.*

Keywords: *Commercial formulates, in vitro, Mango, Black spot disease.*

I. Introduction

Mango (*Mangifera indica L.*) is the 'King of fruits' and important fruit of tropical world belongs to the family Anacardiaceae. India produces 70% of the worlds Mangoes (Cazorla *et al.,* 1998). The soil and climatic conditions of India are highly suitable for Mango cultivation. Karnataka state has occupied 7th place regarding fruit crops with an area of 2.78 lakh hectares and production of 47.36 lakh tons. Kolar and Chitradurga districts are the major mango producing districts in Karnataka (Anon, 2009). The epidemiological characteristics are highly dependent on host range and the emergence of new diseases is some times correlated with broadened host ranges. *Xanthomonads* have the particularity of on extremely narrow host range (Ngoc *et. al.,* 2009). This disease threatens to reduce fruit quality, to cause premature leaf and fruit abscission (Cubero, 2001), Leaves of terrestrial plants provide favorable habitats for colonization by a diversity of bacterial species (Hirano and Upper, 1993). The crop losses more than 80% can occur (Dayakar and Gnanamanickam 1996), fruit and leaf spotting have been observed in mango trees of different cultivars grown in kolar and chitradurga district. Studies on Xcm chemical control are different variable results. With respect to standard bactericides, secondary infection spread of the pathogen in the field can only be reduced by treating seedlings with streptomycin and copper compounds (Hausbeck et al., 2000). The aim of this study was to (i) screen in vitro a range of antimicrobial agents and their effect against Xcm; in laboratory conditions.

II. Materials and Methods

Bacterial cultures, media and growth conditions

Four strains of Xcm were used: CTA-2, CTA- 6, KOL-6 and KOL- 7 (isolated and identified in the microbiology laboratory, Kuvempu university, Shankaraghatta) and strains were cultured in Yeast extract nutrient agar (YNA: yeast extract, 3 g; peptone, 5 g; sodium chloride, 5 g; agar, 20 g; in 1 l of distilled water) and incubated at 30^{0}C. Yeast extract nutrient broth (YNAB: yeast extract, 3 g; peptone, 5 g; sodium chloride, 5 g; in 1 l of distilled water) was used for liquid cultures.

In vitro conditions

Six commercial formulates was evaluated against four strains of Xcm (CTA-2, CTA-6, KOL-6 and KOL-7). The antimicrobial substances used were copper sulphate, copper oxychloride, streptocycline, bactrinashak, mancozeb and bavistin. The MIC and MBC were determined by the broth macrodilution method (Peterson and Shanholtzer, 1992) in 2 ml of YNAB. For copper sulphate, copper oxychloride, streptocycline, mancozeb, bactrinashak concentrations were prepared at 1, 3, 5, 8, 10, 12, 15, 20, 40, 60 and 100, 150, 250 and 400 µl/ml concentrations were prepared, for bavistin up to 1000 µl/ml concentrations were prepared, For controls double distilled sterile water used. The starting bacterial inoculum was 1–5x106 cfu ml-1, and bacterial populations were monitored at 0, 24, 48 and 72 h by cfu counts on YNA plates. The MIC and MBC of compound at which growth was inhibited ≥99.9%, after 48 h of incubation at 30^0C, and sub-inhibitory concentrations assayed (1/2 x MIC) done in quadruplicate, combinations in separately for antibacterial effects.

III. Greenhouse conditions

Plant material and inoculations

Experiments were carried out with mango plants susceptible to bacterial spot. Plants were grown under greenhouse conditions, inoculated with a virulent strain of Xcm on one year old plant reaching the four to six fully expanded leaf stage. Cells were obtained from a 48 h culture at 30^0 C in YNAB. Two inoculation systems were used to evaluate chemical treatment effectiveness: (i) foliar-spray inoculation method (18–20 ml per 12 plants) and after inoculation, plants were covered with polyethylene bags for 5 days.

Chemicals treatment and disease assessment

Antimicrobial substances were applied with the following concentrations: copper sulphate and copper oxychloride 100 µg/ml, bavistin 1000 µg/ml, Streptocycline, mancozeb and bactrinashak for 20 µl/ml were applied half dosage. Chemicals were sprayed on 12 mango plants per treatment until runoff (approximately 300 ml),

IV. Results

Commercial formulates effect of on Xcm growth

The six chemical compounds MIC and MBC values of tested are listed in Table 1. All compounds inhibited bacterial growth after 24 hours incubation, except bavistin which was not active at(1000 µl/ml) concentration. Similarly all formulates were for copper sulphate and copper oxychloride 100 µg/ml, Streptocycline, mancozeb, bactrinashak 20 µl/ml. The MBC values below 2xMIC for copper sulphate, copper oxychloride, streptocycline, bactrinashak, mancozeb and bavistin 3x MIC, addition of these compounds at MIC values produced a >4-log10 reduction in growth of bacterial cultures after 24 h of incubation, but after 48 h incubation, a slight regrowth of the cultures was observed for bavistin. The mixed compounds was evaluated in pairs at sub-inhibitory concentrations (1/2 x MIC), the following combinations compared separately: copper oxychloride + copper sulphate; streptocycline + bactrinashak; mancozeb + copper oxychloride, mancozeb + bavistin and bavistin + bactrinashak. For those combinations, sub inhibitory concentrations of each component drastically reduced cfu counts (>99.9%) from the initial inoculum (Table 2).

Bactericidal effects on mango plants inoculated by spraying

The five compounds out of 6 evaluated in vitro to reduce symptoms caused by Xcm was evaluated in plants sprayed with the pathogen (Table 3). Symptoms, like black spots, appeared in inoculated plants 1 week after inoculation, among inoculated controls and plants treated with bavistin, some displayed leaf spots. copper oxychloride + copper sulphate; streptocycline + bactrinashak; mancozeb + copper oxychloride, mancozeb + bavistin and bavistin + bactrinashak significantly reduced ($p<0.05$) the percentage of affected leaves, The more reduced leaf symptoms by applying streptocycline + copper sulphate and by streptocycline in both trials (Table 3). These compounds reduced black spots, compared with inoculated controls. For copper oxychloride + copper sulphate; streptocycline + bactrinashak; mancozeb + copper oxychloride, mancozeb + bavistin and bavistin + bactrinashak this reduction was significant in the two trials. Xcm was not easily isolated more than 70% of inoculated plants in samples obtained from symptomatic leaves and black spots, which was treated with copper oxychloride + copper sulphate; streptocycline + bactrinashak; mancozeb + copper oxychloride, mancozeb + bavistin and bavistin + bactrinashak treatment in two independent experiments (Table 3).

V. Discussion and Conclusion

Bacterial black spot disease of mango characterized by a rapid expansion of necrotic lesions in buds and leaves and fruits. It is caused by *Xanthomonas campestris* pv. *mangiferaeindicae*. This pathogen usually produces leaf necrosis damage of dormant buds is the most destructive phase of the disease in growing areas Kolar and Chitradurga districts.

The studies on the survival of *Xanthomonas campestris* pv. *mangiferaeindicae*, the incitant of bacterial canker of mango on different weeds present in mango orchards were carried out. Different hosts were collected the bacterium was isolated on nutrient and SX media. The study revealed that the pathogen survives epiphytically on weed hosts which may play an important role in its disease cycle. The presence of the pathogen in the alternative host varied with season (Kishun and Chand, 1988).

The antibacterial compounds are playing a major role in controlling bacterial plant diseases. The commercial bactericides have not been assessed against the causal agent of the disease. The present results of this study, obtained in vitro, showed that the antibiotics ciproflaxacin, tetracycline and kanamycin was the strongest effect against the four tested strains of *Xanthomonas campestris* pv. *mangiferaeindicae* whereas vancomycin was not effective at a concentration of 1000 µgml-1. Other compounds like gentamycin, chloromphenicol, copper sulphate, copper oxychloride and commercial bactrinashak also exerted in vitro antibacterial activity (Thirumalesh, et al., 2011).

A selection of commercial compounds was made based on the in vitro results for in vivo assays in greenhouse-grown mango plants inoculated by spraying. Typical reproducible symptoms were visible 1 week after inoculation. In vivo assays revealed that Spray inoculation produced superficial infection in the early stages, the formulates bactrinashak and copper oxychloride. By contrast, streptocycline did reduce leaf symptoms produced by spray inoculation in two independent trials, and the black spots on the leaves and stems in one. *Bacillus subtilis* were considerably reduced in the field by the application of the antagonist (Okigbo and Osuinde, 2003), differences in disease control according to the inoculation method (Hausbeck et al. 2000) reported that streptomycin applied to seedlings inoculated by misting increased their survival after transplant and prevented severe disease symptoms from developing in the field, Streptocycline was best chemical for control (Mishra and Prakash, 1992). In our study, streptomycin was used as a positive control.

Our study revealed that copper sulphate combined with streptocycline, bactrinashak and copper sulphate alone were the most effective treatments in reducing symptoms in plants inoculated with Xcm by spraying. Products containing copper has reported to significantly reduce foliar leaf, fruit spotting produced by this pathogen (Gleason et al., 1993). Copper treatments were more active when mixed with mancozeb, suggesting a synergistic effect because mancozeb alone did not reduce populations or spread (Hausbeck et al., 2000). Such enhanced activity has also been reported on *Pseudomonas syringae* pv. *mango* when copper is combined with carbamate fungicides. Relevant data from this study was synergistic effects of streptocycline +copper sulphate against Xcm. Both compounds combinations of at half concentration gives significantly reduced bacterial symptoms than copper sulphate alone or streptocycline or bactrinashak alone, treatment with bavistin alone was did not significantly reduce disease symptoms. Using of copper applications to crops lead to contamination in soil, it pollutes soil environment (Ninot et al., 2002), and copper tolerance of plant-pathogenic bacteria increased (Andersen et al., 1991). Consequently, copper applications on commercial crops should be reduced (Ninot et al., 2002). Our results show that copper sulphate at reduced dosages in combination with streptocycline, bactrinashak or alone may be useful as a protective compound to prevent the pathogen spreading in greenhouse.

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Table 1: Minimal inhibitory and bactericide concentrations (MIC and MBC) of chemical compounds against four strains of *X. campestris* pv. *mangiferaeindicae*

Chemical compounds	**Strains of *X. campestris* pv. *mangiferaeindicae***			
	CTA-2	**CTA- 6**	**KOL- 6**	**KOL- 7**
	MIC/MBC[a]	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**
Copper sulphate	100/ >200	100/ >200	100/ >200	100/ >200
Copper oxychloride	100/ >400	100/ >400	100/ >400	100/ >400
Streptocycline	20/40	20/40	20/40	20/40
Mancozeb	20/40	20/40	20/40	20/40
Bactrinashak	20/40	20/40	20/40	20/40
Bavistin	500/ >1000	500/ >1000	500/ >1000	500/ >1000

[a] MIC and MBC are expressed in µl/ ml

Table 2. Effect of individual chemical compounds and combinations at sub-inhibitory concentrations (1/2 x MIC) that showed in vitro synergistic effect on *X. campestris* pv. *mangiferaeindicae*, after 24 h of incubation

Initial inoculums (cfu ml^{-1})[a]	**Combination of chemical compounds**	**Recovered cells (cfu ml^{-1})**[a]
$1.17\pm0.09x\ 10^6$	½ Copper oxychloride (50 µl/ ml)	$6.32\pm2.01x10^9$
	½ Copper sulphate (50 µl/ml)	$6.71\pm1.72x10^9$
	½ Copper oxychloride + ½ Copper sulphate	$1.13\pm0.92x10^2$
	½ Streptocycline (10 µl/ml)	$2.68\pm1.02x10^9$
	½ Bactrinashak (10 µl/ml)	$6.68\pm6.81x10^9$
	½ streptocycline + ½ Bactrinashak	3.45±2.61x10
	½ mancozeb (10 µl/ml)	$8.48\pm2.02x10^9$
	½ Copper oxychloride (50 µl/ml)	$6.48\pm7.58x10^9$
	½ mancozeb + ½ Copper oxychloride	7.48±2.60x10
	½ mancozeb (10 µl/ml)	$10.48\pm2.02x10^9$
	½ bavistin (600 µl/ml)	$17.28\pm6.85x10^9$
	½ mancozeb + ½ bavistin	13.50±5.60x10
	½ bavistin (600 µl/ml)	$26.48\pm2.02x10^9$
	½ Bactrinashak (10 µl/ml)	$7.88\pm7.85x10^9$
	½ bavistin + ½ Bactrinashak	14.50±5.60x10

a values presented are means (±SE) for four repetitions.

Table 3. Effect of chemical treatments on mango leaf symptoms, presence of leaf spots and isolation of *X.campestris* pv. *mangiferaeindicae* from leaves after spray inoculation of *X.campestris* pv. *mangiferaeindicae* (two greenhouse trials)

Treatments[a]	**Symptomatic leaves (%)**[b,c]		**Pathogen isolation**[d]	
	Trial 1	**Trial 2**	**Trial 1**	**Trial 2**
Uninoculated control [e]	0.0	0.0	0/12	0/12
Inoculated control	71.1a	65.6ab	12/12	12/12
Copper sulphate	63.6ab	65.9a–c	6/12	5/12
Copper oxychloride	71.3h	58.1e	7/12	8/12
Streptocycline	63.6bc	63.6ab	5/12	4/12
Mancozeb	70.2h	68.1e	10/12	9/12
Bactrinashak	56.1de	60.1a	4/12	4/12
Bavistin	87.3h	78.1e	11/12	12/12
Streptocycline + Copper sulphate	62.6i	61.7f	2/12	3/12
Copper sulphate + bactrinashak	65.6b	59.6cd	3/12	4/12
Mancozeb+ Copper oxychloride	59.2d	69.2b–d	7/12	9/12
Bavistin + Streptocycline	59.2f	55.2a–c	7/12	9/12
Streptocycline+ Bactrinashak	47.3h	43.1e	3/12	4/12

a. Treatments were applied twice, 5 d before and 6 d after inoculation with the pathogen at the doses indicated in the text.
b. Values are the mean of 12 plants per treatment. Numbers followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($p < 0.05$).
c. Data from percentage of symptomatic leaves were transformed to angular (Y= arcsine $[\%]^{1/2}$) for analysis of variance.
d. Positive isolation of the pathogen from samples obtained from the internal tissues of 12 mango plants.
e. Values equal to zero obtained from the uninoculated control were excluded from the analyses of variance.