Recent Research in Science and Technology 2011, 3(9): 57-61
ISSN: 2076-5061
www.scholarjournals.org

www.recent-science.com

RRST-Microbiology

Assessment of Chemical Compounds for *in vitro* and *in vivo* Activity against Bacterial Black Spot of Mango

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Article Info

Article History

Received : 27-05-2011
Revised : 25-07-2011
Accepted : 04-08-2011

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Editor: M.B. Hiremath

Abstract

Bacterial black spot of mango caused by *Xanthomonas campestris* pv. *mangiferaeindicae* (Xcm) produces considerable economic losses in many parts in india. The extent to which bactericides control this disease effectively is low. In this study the bactericidal effect of different products was assessed in vitro and in vivo in mango plants under greenhouse conditions. Thirteen antibacterial substances, one commercial formulate and also combinations were tested. In vitro analysis showed that minimal inhibitory concentration (MIC) of antibacterial substances was between 2–8 µgml^{-1}, except for copper sulphate with a MIC value of 100 µgml^{-1}and Vancomycin, Amoxycillin which was not active at 1000 µgml^{-1}. MIC values of commercial formulate bactrinashak ranged between 5 and 30 µgml^{-1}, and combinations of ciprofloxacin + copper sulphate; ciprofloxacin + bactrinashak; ciprofloxacin + copper oxychloride, ciprofloxacin + tetracycline and tetracycline + bactrinashak, showed a great effect at sub-inhibitory concentrations. Treatments including copper sulphate and copper oxychloride significantly reduced disease symptoms on plants, whereas bacitracin was less effective, where as fluconozole and penicillin does not show any inhibition. In two different field trials, the percentage of leaves symptoms was lower, after treatment with copper sulphate combinations than in inoculated controls. These combinations of different antibacterial substances results were better than copper sulphate alone. We conclude that the combination of copper sulphate with ciproflaxacin may be useful in controlling symptoms of this disease in greenhouses.

Key Words: Antibiotics, Copper sulphate, Mango, *Xanthomonas campestris* pv. *Mangiferaeindicae*

Introduction

Bacterial black spot of mango caused by *Xanthomonas campestris* pv. *mangiferaeindicae*(Xcm) is one of the most important bacterial disease causing mango crops, in karnataka. Mango is cultivated through out the tropics as well as subtropical areas and is threatened by several destructive diseases (Pruvost et al., 2009). When the infection occurs after epiphytic spread of the pathogen, marginal necrosis of leaves and small black spots are common, and lesions, occasionally appear on fruits (Carlton et al., 1998). When the disease symptoms increases lesions spread to stems, petioles and flowers, yield reductions can result from the reduced photosynthetic capacity of infected foliage, leaf defoliation and flower abortion (Ji et al, 2006). Disease symptoms include necrosis of vegetative and flower buds and bud failure before bud break (Cozorla, 1998), Then crop becomes infected with the bacteria. Present control measures for mango fruit disease include extensive pre-harvest spraying with copper oxychloride (Silimela & Korsten, 2007), then adopted to reduce losses and prevent secondary infection in order to decrease epiphytic pathogen populations (Carlton et al., 1994). Major strategies for disease management have included pre and post harvest fungicide application (Wellington, 2003). Biocontrol employing different bacteria (Umesha, 2006; Govender & Korsten, 2006) have been proposed to improve control strategies. 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 synergistic effect against Xcm; and (ii) determine in vivo to what extent they effectively control the pathogen under greenhouse conditions.

Materials and methods

Bacterial cultures, media and growth conditions

Four strains of Xcm were used: MYS-2, CTA- 6, CHK- 2 and KOL- 4 (isolated 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 30^0C. 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

Thirteen antimicrobial substances and one commercial formulates was evaluated against four strains of Xcm (MYS-2, CTA- 6, CHK- 2 and KOL- 4). The antimicrobial substances used were copper sulphate, copper oxychloride, tetracycline, streptomycin, chloromphenicol, bacitracin, ciprofloxacin, gentamicin, fluconozole, penicillin, vancomycin, amoxicillin and kanamycin (himedia, mumbai, india), and the commercial formulates: bactrinashak (2-bromo-2-nitro propane-13-diol, indofil chemicals company, mumbai, india). The MIC and MBC were determined by the broth macrodilution method (Peterson and Shanholtzer, 1992) in 2 ml of YNAB. Antimicrobial substances dissolved in sterile water and added to YNAB at concentrations of 1, 2, 4, 6, 8, 10, 12, 15 and 20 μgml^{-1}. For copper sulphate of 50, 80, 100, 150, 250 and 400 μgml^{-1} concentrations were prepared, for vancomycin, fluconozole and penicillin up to 1000 μgml^{-1} concentrations were prepared. Commercial formulate bactrinashak concentrations were prepared at 1, 3, 5, 8, 10, 12, 15, 20, 40, 60 and 100 $\mu l\ ml^{-1}$. For controls double distilled sterile water used. The starting bacterial inoculum was $1–5x10^6$ 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 30^0, and sub-inhibitory concentrations assayed (1/2 and 1/4 x MIC) done in quadruplicate, combinations in separately for synergistic effects.

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 300 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 (ii) pricking inoculation method with an insulin needle. 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 μgml^{-1}, vancomycin, fluconozole and penicillin 1000 μgml^{-1}, Streptomycin, gentamycin, chloromphenicol, kanamycin 4 μgml^{-1}, ciproflaxacin and tetracycline 2 μgml^{-1}, amoxycillin, bacitracin 8 μgml^{-1}, commercial formulate bactrinashak for 20 μlml^{-1} were applied half dosage. Chemicals was sprayed on 12 mango plants per treatment until runoff (approximately 300 ml), using a hand-held spray. Two applications were made per treatment, the first 5 days before and the second 6 days after pathogen inoculation. After spray, plants were allowed to dry until no liquid droplets were visible. Control plants were sprayed with the same volume of sterile tap water. Experiments were conducted in 2008, 2009 and 2010.

Symptoms caused by Xcm in mango plants were evaluated 6 weeks after inoculation, the presence of black spots on the leaves in the inoculated area (Gleason et al.1993) rated on a scale from 0 to 3, where 0 = no lesions, 1 = <5 small and dispersed spots, 2 = 45 dispersed spots, and 3 = black spots occupying all inoculated areas. Samples of leaves next to black spots were taken for pathogen isolation. In plants inoculated by pricking, the percentage of infected leaves was calculated to assess disease severity. To confirm the isolated pathogen streaked on YNA plates, then incubated for 2 d at 30^0C.

Statistical analysis

Analysis of variance (ANOVA) was performed and means were separated by Fisher's least significant difference test at $p<0.05$. Data for percentage of black spot and incidence of necrosis on leaves were subjected to angular transformation ($Y = \text{arcsine } [\%]^{1/2}$) to stabilise variance.

Results

Antimicrobial compounds effect of on Xcm growth

The 13 chemical compounds MIC and MBC values of tested are listed in Table 1. All compounds inhibited bacterial growth after 24 hours incubation, except vancomycin, fluconozole and penicillin, which was not active at(1000 $\mu g\ ml^{-1}$) concentration. Similarly streptomycin, gentamycin, chloromphenicol, kanamycin (MIC 4 μgml^{-1}), ciproflaxacin and tetracycline (MIC 2 μgml^{-1}), amoxycillin, bacitracin (MIC 8 μgml^{-1}), exhibited low MIC values, whereas copper sulphate showed (MIC 100 μgml^{-1}) concentration. The MICs of commercial formulates were 20 μlml^{-1} for bactrinashak.

The MBC values below 2xMIC for Chloromphenicol, streptomycin, gentamicin and kanamycin and for amoxicillin, bacitracin 3x MIC, addition of these compounds at MIC values produced a >4-$\log_{10}$ 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 amoxycillin and bacitracin. The mixed compounds was evaluated in pairs at sub-inhibitory concentrations (1/2 and 1/4 x MIC), the following combinations compared separately: ciprofloxacin + copper sulphate; ciproflaxacin + bactrinashak; tetracycline + bactrinashak, ciprofloxacin + copper oxychloride and ciprofloxacin + tetracycline. For those combinations, sub inhibitory concentrations of each component drastically reduced cfu counts (>99.9%) from the initial inoculum (Table 2).

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*			
	MYS-2,	CTA- 6,	CHK- 2	KOL- 4
	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
Kanamycin	4/ 8	4/ 8	4/ 8	4/ 8
Tetracycline	2/ 4	2/ 4	2/ 4	2/ 4
Streptomycin	4/ 8	4/ 8	4/ 8	4/ 8
Chloromphenicol	4/6	4/6	4/ 6	4/ 6
Bacitracin	8/18	8/18	8/18	8/18
Ciprofloxacin	2/4	2/4	2/4	2/4
Gentamicin	4/8	4/8	4/8	4/8
Fluconozole	1000/5000	1000/5000	1000/5000	1000/5000
Penicillin	1000/5000	1000/5000	1000/5000	1000/5000
Vancomycin	1000/4000	1000/4000	1000/4000	1000/4000
Amoxicillin	8/18	8/18	8/18	8/18
Bactrinashak	20/40	20/40	20/40	20/40

[a] MIC and MBC are expressed in µg ml^{-1} except for commercial compounds bactrinashak, which are in µl ml^{-1}

Table 2: Effect of individual chemical compounds and combinations at sub-inhibitory concentrations (1/2 and 1/4 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±0.09x 10^6	½ Ciprofloxacin (1 µg ml^{-1})	6.32±2.01x10^9
	½ Copper sulphate (50 µg ml^{-1})	6.71±1.72x10^9
	½ Ciprofloxacin + ½ Copper sulphate	1.13±0.92x10^2
	½ Ciprofloxacin + ¼ Copper sulphate	1.08±2.61x10
	¼ Ciprofloxacin + ½ Copper sulphate	2.58±0.19x10^3
	¼ Ciprofloxacin + ¼ Copper sulphate	7.75±3.75x10
1.17±0.09x 10^6	½ Ciprofloxacin (1 µg ml^{-1})	6.68±1.02x10^9
	½ Bactrinashak (10 µl ml^{-1})	8.68±6.81x10^9
	½ Ciprofloxacin + ½ Bactrinashak	3.45±2.61x10
	½ Ciprofloxacin + ¼ Bactrinashak	2.25±2.60x10
	¼ Ciprofloxacin + ½ Bactrinashak	3.78±7.47x10^2
	¼ Ciprofloxacin + ¼ Bactrinashak	3.52±1.56x10
1.16±0.07x 10^6	½ Ciprofloxacin (1 µg ml^{-1})	5.48±2.02x10^9
	½ Copper oxychloride (50 µg ml^{-1})	7.48±7.58x10^9
	½ Ciprofloxacin + ½ Copper oxychloride	3.75±2.60x10
	½ Ciprofloxacin + ¼ Copper oxychloride	4.50±2.80x10
	¼ Ciprofloxacin + ½ Copper oxychloride	3.38±3.47x10^2
	¼ Ciprofloxacin + ¼ Copper oxychloride	7.76±5.30x10^5
1.16±0.07x 10^6	½ Ciprofloxacin (1 µg ml^{-1})	6.48±2.02x10^9
	½ Tetracycline (2 µg ml^{-1})	7.28±6.85x10^9
	½ Ciprofloxacin + ½ Tetracycline	3.50±5.60x10
	½ Ciprofloxacin + ¼ Tetracycline	4.25±2.80x10
	¼ Ciprofloxacin + ½ Tetracycline	4.08±1.45x10^2
	¼ Ciprofloxacin + ¼ Tetracycline	5.48±4.36x10^5
1.17±0.09x 10^6	½ Tetracycline (2 µg ml^{-1})	6.48±2.02x10^9
	½ Bactrinashak (10 µl ml^{-1})	7.88±7.85x10^9
	½ Tetracycline + ½ Bactrinashak	3.55±8.60x10
	½ Tetracycline + ¼ Bactrinashak	2.72±6.20x10
	¼ Tetracycline + ½ Bactrinashak	4.58±2.47x10^2
	¼ Tetracycline + ¼ Bactrinashak	4.25±4.65x10

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	68.6ab	65.9a–c	7/12	5/12
Copper oxychloride	71.3h	58.1e	7/12	8/12
Ciproflaxacin	59.1de	68.1a	4/12	5/12
Kanamycin	64.3g	67.1d	5/12	7/12
Tetracycline	74.7cd	64.5a–d	8/12	7/12
Gentamycin	71.3ab	58.2a–c	12/12	11/12
Streptomycin	63.6bc	59.6ab	9/12	10/12
Chloromphenicol	70.2h	68.1e	10/12	9/12
Bacitracin	57.3h	58.1e	11/12	12/12
Fluconozole	57.3h	58. 1e	12/12	12/12
Penicillin	61.5h	51.6e	12/12	12/12
Vancomycin	58.4ab	61.5d	12/12	12/12
Amoxicillin	67.3h	58.5e	11/12	11/12
Bactrinashak	70.8h	78.5e	7/12	8/12
Ciproflaxacin+copper sulphate	62.6i	61.7f	2/12	3/12
Ciproflaxacin+Bactrinashak	67.6b	51.6cd	8/12	7/12
Ciproflaxacin+copper oxychloride	53.2d	62.2b–d	7/12	10/12
Ciproflaxacin+Tetracycline	49.2f	45.2a–c	8/12	7/12
Tetracycline+Bactrinashak	47.3h	38.1e	7/12	8/12

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 ($Y = \arcsin [\%]^{1/2}$) 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.

Bactericidal effects on mango plants inoculated by spraying and pricking

The seven compounds out of 13 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 bacitracin, amoxycillin and gentamycin, some displayed leaf spots. Copper sulphate, streptomycin, tetracycline, ciproflaxacin+copper sulphate and tetracycline + copper sulphate, cipriflaxacin + copper oxychloride and ciprofloxacin+ bactrinashak significantly reduced ($p<0.05$) the percentage of affected leaves in both assays, whereas kanamycin, bactrinashak, tetracycline + ciproflaxacin and streptomycin + bacitracin did so only in one. The more reduced leaf symptoms by applying ciproflaxacin + copper sulphate (83.4% and 77.1%) and by ciproflaxacin (66.7% and 58.3%) in both trials (Table 3). These compounds reduced black spots, compared with inoculated controls. For ciproflaxacin, bactrinashak, streptomycin + bactrinashak, and streptomycin + chloromphenicol, this reduction was significant in only one of the two trials. Xcm was easily isolated more than 70% of inoculated plants in samples obtained from symptomatic leaves and black spots, except ciproflaxacin and the combination ciproflaxacin + copper sulphate treatment in two independent experiments (Table 3).

The compounds inoculated by pricking, and combinations of compounds at sub inhibitory concentrations given good results in laboratory conditions. After 5 weeks, inoculated mango plants developed black spot at the inoculation area, the assayed compounds or their combinations did not showed significant reduction of leaf spots compared with untreated plants. Copper sulphate, ciprofloxacin, bactrinashak, tetracycline and the combination of ciproflaxacin + bactrinashak significantly reduced ($p<0.05$).

Discussion and Conclusion

The antibacterial compounds are playing a major role in controling 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 $\mu g ml^{-1}$. Other compounds like gentamycin, chloromphenicol, copper sulphate, copper oxychloride and commercial bactrinashak also exerted in vitro antibacterial activity.

A selection of antimicrobial compounds was made based on the in vitro results for in vivo assays in greenhouse-grown mango plants inoculated by spraying and pricking. For both inoculation methods, typical reproducible symptoms were visible 1 week after inoculation. In vivo assays revealed that pricking inoculation with Xcm produced symptoms of systemic infection. Spray inoculation produced superficial infection in the early stages, whereas the leaf tissues of plants inoculated by pricking were invaded very rapidly, preventing treatment efficacy, even of the antibiotics ciproflaxacin and tetracycline.

By contrast, streptomycin 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 ciproflaxacin 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 ciproflaxacin +copper sulphate against Xcm. Both compounds combinations of at half concentration gives significantly reduced bacterial symptoms than copper sulphate alone or ciproflaxacin alone, which 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 ciproflaxacin or alone may be useful as a protective compound to prevent the pathogen spreading in greenhouse.

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