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Invitro Evaluation of Chemical Formulates on *Xanthomonas axonopodis pv. punicae*

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A B S T R A C T

Pomegranate is important fruit for its nutritional, medicinal and ornamental properties and its high consumption and industrial value. Bacterial blight of Pomegranate is caused by *Xanthomonas axonopodis* pv. *punicae* strongly damaged the pomegranate production. In this study the bactericidal effect of different chemicals were assessed in six commercial formulate and combinations are tested. *In vitro* analysis showed that minimal inhibitory concentration (MIC) of copper sulphate, copper oxychloride with a MIC value of 100 µl/ml and MIC values of commercial formulate bactrinashak ranged between 0 and 30 µg/ml, and combinations of copper oxychloride + copper sulphate; streptocycline + bactrinashak; K-cycline + copper oxychloride, and K - cycline + bactrinashak, showed a great effect at sub-inhibitory concentrations. The study conclude that the combination of copper sulphate with streptocycline, bactrinashak, K-cycline and X-traoralone useful as a protective compound to prevent the pathogen in laboratory conditions.

Introduction

Pomegranate (*Punica granatum*), being a favourite table fruit of tropical and subtropical regions of the world, has emerged as commercial fruit in many Indian states including Maharashtra, Andhra Pradesh, Uttar Pradesh, Gujarat, Rajasthan, Karnataka and Tamil Nadu (Mondal and Mani, 2009). Bacterial diseases cause substantial loss to the productivity of major crop plants. Unlike fungal diseases, bacterial diseases not controleffectively through chemical methods (Mondal and Shanmugam, 2013). However, bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Xap) strongly damaged the pomegranate production, inducing large economic losses to the Indian growers (Mondal and Sharma 2009).The disease was of minor importance until the recent appearance of the *Xap* in epidemic form in

many pomegranate growing states of India, including Karnataka and Maharashtra. The bacterial blight of pomegranate is known to be confined mainly in India (Chand and Kishun, 1991).Several management options have been investigated. Among them application of antibiotics, chemicals and other cultural practices. Treatments by chemicals had limited success against the disease (Kumar, *et al*. 2009). Bacterial blight is one of the most devastating diseases of pomegranate occurring in major pomegranate growing states of India. Bacterial blight has been observed damaging the pomegranate crop in moderate to severe proportion resulting in enormous losses. The infected plant parts are the potential sources of primary inoculums to spread the disease. Attempts have been made in India to control this disease by either uprooting the whole plant or spraying chemicals/antibiotics (Manjula and Khan, 2002), but complete control has not been achieved so far. Studies on *Xap* 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 streptocycline and copper compounds. The aim of this study was to screen *in vitro* a range of antimicrobial agents and their effect against Xap; in laboratory conditions.

Materials and Methods

Bacterial Cultures, Media and Growth Conditions

Isolation and Identification of *Xap*

Bacterial blight infected leaves and fruits (Fig.1) were collected from the pomegranate fields of Challakere Taluk, Chitradurga District, Karnataka, India. The infected leaf were cut into 2-3 cm pieces and sterilized by 70% ethanol for 30 min followed by 2-3 times distilled water wash. Then they were squeezed with fine forceps to release the pathogenic extracts and then the crude extracts were directly cultured on nutrient agar and Glucose yeast chalk agar(GYCA) (Petersen, 2010) plates with 1% NaCl and kept for incubation at 28^0C for 24 to 72 hour. After incubation the yellow colour colonies were isolated. Identification and characterization of the bacterial blight pathogen was carried out by subjecting to various biochemical tests, such as Gram staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test, starch hydrolysis, Lipase activity and Arginine dehydrogenase test (Lelliot and Stead, 1987), gelatin hydrolysis, and catalase tests. Three strains of *Xap* were used: Banjagere-3(BAN-3), Challakere-1(CLK- 1), and Nayakanahatty-3(NYH- 3) strains were selected and cultured for further analysis.

In vitro Conditions

Six commercial formulates was evaluated against three strains of Xap (BAN-3, CLK-1, and NYH-3). The antimicrobial substances used were copper sulphate, copper oxychloride, streptocycline, bactrinashak, K- cyclineandX-tra. The MIC and MBC were determined by the broth macrodilution method (Peterson and Shanholtzer, 1992) in 2 ml of GYCA. For copper sulphate, copper oxychloride, streptocycline, bactrinashak, K- cycline and X-tra concentrations were prepared at 1, 3, 5, 8, 10, 12, 15, 20, 40, 60 and 100, 150, 250 and 400 µl/ml concentrations, 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 GYCA broths. The MIC and MBC of compound at which growth after 24 to 48 h of incubation at 28^0C, and sub-inhibitory concentrations assayed in combinations for their antibacterial effect.

Results and Discussion

Isolation and Identification of *Xap*

The isolated yellow colour colonies were identified and characterized of the bacterial blight pathogen by various biochemical tests, such as Gram staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test, starch hydrolysis, Lipase activity and Arginine dehydrogenase test (Lelliot and Stead, 1987), gelatin hydrolysis, and catalase tests and pathological tests. The obtained results were compared with authentic *Xanthomonas* results.

Sensitivity Effect on *Xap* 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, all formulates were copper sulphate and copper oxychloride 100 µg/ml, Streptocycline, bactrinashak, K-Cyclineand X-tra 20 µl/ml.

The MBC values below 2xMIC for copper sulphate, copper oxychloride, streptocycline, bactrinashak, K- Cycline and X-tra, addition of these compounds at MIC values produced reduction in growth of bacterial cultures after 24 h of incubation, The mixed compounds was evaluated in combinations at sub-inhibitory concentrations (1/2 x MIC), the following combinations compared separately: copper oxychloride + copper sulphate; streptocycline + bactrinashak; K-Cycline + copper oxychloride,K- Cycline + X-traand K- Cycline + bactrinashak. For those combinations, sub inhibitory concentrations of each component drastically reduced cfu counts from the initial inoculum (Table 2 and fig-2) and distilled water served as control.

In the present study, field survey was undertaken in the major pomegranate growing region of Chitradurga district, challakere taluk and the study revealed that the bacterial blight disease prevails in up to 90.0% orchards. Bacterial blight of pomegranate was found to be highly destructive, wide spread disease and a threat to pomegranate production due to its high epidemic potential. The antibacterial compounds are spraying in controlling bacterial diseases. The commercial bactericides control under *in vitro* six chemical treatments showed effective response to each isolate. Combination of streptocycline (100-300 ppm) with copper oxychloride (0.3%) was reported to be effective against *X. citri* (Kale *et al.*, 1994). Desai *et al*. (1967) and Raj and Moniz (1967) had reported effectiveness of streptocycline against *Xanthomonas* sp. *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 in control (Mishra and Prakash, 1992).

Acibenzolar-S-methyl is a systemic inducer of resistance in plants (Kessmann *et al.*, 1994). Application of acibenzolar-S-methyl reduced the incidence of diseases caused by different pathogens such as bacterial diseases (Brisset *et al.*, 2000). Rego *et al*. (2006) obtained similar *in vitro* results when these two methods were used to evaluate fungicides to control *C. liriodendri*. Some of the fungicides used in our experiments were also previously evaluated *in vitro* by Halleen *et al*. (2007). *In vitro* inhibition of Gram-positive bacteria has also been reported (Hinton and Ingram, 2003).

Table.1 Effect of Chemical Compounds against Three Strains of *X. axanopodis pv. punicae*

Sl.No.	Chemical compounds / antibiotics	Strains of *X. axanopodis* pv. *punicae*[a]		
		BAN-3	CLK-2	NYH-3
		MIC/MBC	MIC/MBC	MIC/MBC
1	Control	0/0	0/0	0/0
2	Copper sulphate	100/ >200	100/ >200	100/ >200
3	Copper oxychloride	100/ >200	100/ >200	100/ >200
4	Streptocycline	20/40	20/40	20/40
5	Bactrinashak	20/40	20/40	20/40
6	K- cycline	20/40	20/40	20/40
7	X- tra	20/40	20/40	20/40

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

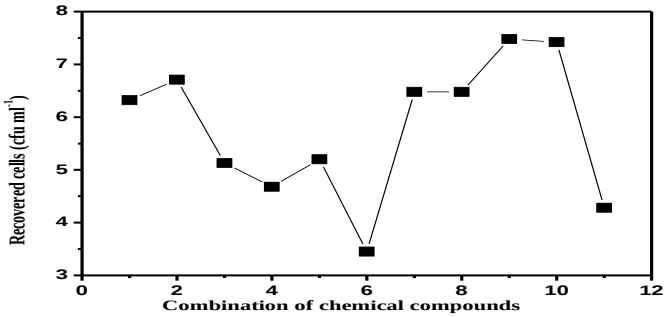
Table2 *In vitro* Synergistic Effect on *Xap*, after 24 h of Incubation

Sl.No.	Initial inoculums (cfu ml^{-1})[a]	Combination of chemical compounds	Recovered cells (cfu ml^{-1})[a]
1	$1.12\pm0.0x\ 10^6$	½ Copper oxychloride (50 µl/ ml)	$6.32\pm2.01x10^9$
2		½ Copper sulphate (50 µl/ml)	$6.71\pm1.72x10^9$
3		½ Copper oxychloride +½ Copper sulphate	$5.13\pm0.92x10^2$
4		½ Streptocycline (10 µl/ml)	$4.68\pm1.02x10^9$
5		½ Bactrinashak (10 µl/ml)	$5.20\pm6.81x10^9$
6		½ Streptocycline +½ Bactrinashak	$3.45\pm2.61x10$
7		½ K- cycline (10 µl/ml)	$6.48\pm2.02x10^9$
8		½ K- cycline +½ Copper oxychloride	$6.48\pm7.58x10^9$
9		½ X-tra (10 µl/ml)	$7.48\pm2.60x10^9$
10		½ K- cycline +½ X-tra	$7.42\pm2.02x10^9$
11		½ K- cycline+ ½ Bactrinashak	$4.28\pm6.85x10^9$

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

Fig.1 Bacterial Blight on Pomegranate Fruits

Fig.2 *In vitro* Synergistic Effect on *Xap*, after 24 h of Incubation



Our study revealed that copper sulphate and copper oxychloride combined with streptocycline, bactrinashak, K- cycline and X-tra and alone were the most effective treatments in inhibiting the *Xap*. Products containing copper has reported to significantly reduced, fruit spotting produced by this pathogen (Gleason *et al.*, 1993). Copper treatments were more active when mixed with other, 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 *Xap*. Both compounds combinations of at half concentration gives significantly reduced bacteria than copper sulphate alone or streptocycline or bactrinashak, K-cycline and X-traalone. 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 and copper oxychloride at reduced dosages in combination with streptocycline, bactrinashak, K-cycline and X-traor alone proved useful as a protective compound to prevent the blight.

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