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In vitro Prospects of Botanicals, Bioagents and Micronutrients in the Inhibition of *Xanthomonas axonopodis* pv. Punicae Causing Bacterial Blight of Pomegranate

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Pomegranate (*Punica granatum* L.) commonly called a fruit of paradise, is having antioxidant property affected by a dreaded pathogen *Xanthomonas axonopodis* pv. *punicae (Xap)* causing bacterial blight. Among 10 plant extracts tested soapnut recorded highest inhibition zone of 19.75 mm at 10% and 9.5mm at 5% concentration which is followed by garlic and meswak 9.0mm and 8.0mm at 10% and 7.25mm and 7.00mm at 5% concentration respectively. Among five strains of *Pseudomonas fluorescens* tested, strain no. 326 (4) and 139 has recorded highest inhibition zone of 31.0mm and 30.0mm respectively, and followed by strain pf1 (27.75 mm), strain 134 (27.25 mm) and pf5 (26.50 mm). Among the eleven different strains of Pink Pigmented Facultative Methylotrophs (PPFM) tested strain no. 10L and 75L has recorded highest inhibition zone of 9.5mm and 8.0mm respectively. Among nine micronutrients $CuSO_4$ has recorded maximum inhibition zone of 0.60mm at 0.05%, 0.75mm at 0.1% and 0.95mm at 0.2% 12.5mm at 0.5% concentrations respectively.

Key words: Pomegranate, bacterial blight, botanicals bioagents and micronutrients.

The pomegranate, *Punica granatum* L., is regarded as the "Fruit of Paradise" which is extensively cultivated in Spain, Morocco and other countries around the Mediterranean, Egypt, Iran, Afghanistan, Arabia and Baluchistan (Miguel *et al.,* 2010). In India it is grown as a commercial crop in Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Madhya Pradesh and Utter Pradesh. Area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, drought tolerance, higher yield levels with excellent keeping quality and remunerative prices in respect of domestic and export market. Pomegranate is a good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin-C and citric acid. (Malhotra *et al*., 1983). The fruits of pomegranate are known to posses pharmaceutical and therapeutic properties and are used as component of fold medical practices.

Cultivation of pomegranate in recent years has met with different traumas such as pest and diseases. Among the diseases infecting pomegranate, the bacterial blight' caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh) Vauterin *et al.* is as a major threat. It causes loss upto 70-80% (Benagi *et al*.,2012). In Karnataka, survey report revealed that, 20 – 90 per cent of disease severity in Bijapur and Bagalkot districts (Ravikumar *et al*., 2006). Recent reports revealed the highest severity of tree was 74.80 per

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cent in Bagalkot district and minimum severity of 6.73 per cent in Bellary districts (Anon., 2008). During 2008 – 09 the disease has reached its alarming stage bringing substantial damage to the crop and heavy loss to the farmers. In recent years, there has been a major thrust on residue free organic pomegranate production. Plant nutrients also play an important role in susceptibility or resistant mechanism of the host to different pathogens. Taking the task into consideration, efficient botanicals, bioagents and micronutrients need to be explored to fit into the management schedule.

MATERIALS AND METHODS

In vitro evaluation of plant extracts

Plant based pesticides, which are relatively safe, economical and non-hazardous can be used successfully for the management of bacterial diseases in crop plants. The present investigation was aimed at screening seven plant extracts viz., Garlic, Soapnut, Meswak, Adathoda, Tulsi, Nerium, Neem for their antibacterial properties against *Xap*.

Fresh plant materials were collected and washed in distilled water, 100 grams of fresh sample was chopped and macerated in a sterilized pestle and mortar by adding 100 ml of distilled steriled water (1:1 w/v). The extract was filtered through Whatman's No. 1 filter paper; filtrate thus obtained was used as a stock solution. To study the antibacterial mechanism of plant extracts, inhibition zone assay method was followed.

A loopfull (72 hours old) of *Xap*, multiplied on nutrient glucose agar (15ml) in Petriplates was mixed with 5ml distilled sterilized water. 300µl of this solution was spread over nutrient glucose agar. Sterilized filter paper discs (Whatman No. 1) measuring 5 mm diameter were soaked for 10 minutes in 5% and 10% of different plant extracts and placed on the surface of the nutrient glucose agar medium which was spread with *Xap* contained in the Petri plates. The inoculated plates were incubated at 28^0 C for 72 hours.

Observations were recorded by measuring the diameter of the inhibition zone around the filter paper disc in each plant extract at different concentrations in millimeter and data obtained was analysed statistically.

In vitro evaluation of bioagents on the growth of the *Xanthomonas axonopodis* pv. *punicae*

The five *Pseudomonas fluorescens* strains and eleven PPFM strains were obtained from department of molecular biology and biotechnology, Agricultural college, Dharwad and department of agricultural microbiology, Agricultural college, Dharwad, University of agricultural sciences, Dharwad respectively were tested for their inhibition effect on the growth of *X. a.* pv. *punicae* by inhibition zone assay method as mentioned above method.

Pseudomonas fluorescens was grown and maintained on King's B broth and PPFM strains (phylloplane bacteria) on AMS (Ammonium Mineral Salt) broth.

In vitro evaluation of micronutrients on the growth of *Xanthomonas axonopodis* pv. *punicae*

Effect of micronutrients for their ability to inhibit the growth and development of *X. a.* pv. *punicae* and to control the bacterial blight of pomegranate were tested. Following are the nutrients and their concentrations which were tested *in vitro viz.*, Boron, Calcium, Iron, Magnesium, Mmicron special (boron, $MgSO_4$ and $ZnSO_4$), Zinc, Manganese, Sulphur, Copper. Micronutrients were diluted at 0.05, 0.1, 0.20 and 0.5% concentration and inhibition zone assay was done by following the above mentioned method.

RESULTS AND DISCUSSION

In vitro evaluation of plant extracts on the growth of *Xanthomonas axonopodis* pv. *punicae*.

Among the seven plant extracts tested, soapnut was recorded highest inhibitory zone of 1.97cm at 10% and 0.95cm at 5% concentration which is significantly superior over other plant extracts. The extracts of garlic, miswak and adathoda were inhibited the growth of *X. a.* pv. *punicae* producing the inhibitory zone of 0.90cm, 0.80cm and 0.825cm at 10% and 0.72cm, 0.70cm and 0.62cm at 5% concentration respectively. The extracts of neem, nerium and tulsi failed to inhibit the growth of bacterium even at 10% concentration. Among combination of effective botanicals, adathoda + soapnut recorded inhibitory zone of 1.47cm and 1.00cm at 10% and 5% respectively followed by meswak + soapnut and adathoda + meswak (Table 1).

Table 1. Bioefficacy of different plant extracts against *Xanthomonas axonopodis* pv. *punicae* under *in vitro* conditions

Plant extracts	Parts used	Inhibition zone (mean diameter in cm)		
		5%	10%	Mean
Garlic (*Allium sativum*)	Clove	0.73(1.31)*	0.90(1.38)	0.81(1.35)
Soapnut (*Sapindus mukorossi)*	Fruit	0.95(1.40)	1.98(1.72)	1.46(1.57)
Meswak (*Salvadora persica)*	Stem	0.70(1.26)	0.80(1.30)	0.65(1.28)
Adathoda (*Adathoda vessica)*	Leaf	0.63(1.27)	0.83(1.35)	0.73(1.31)
Tulsi (*Ocimum sanctum)*	Leaf	0.00(1.00)	0.00(1.00)	0.00(1.00)
Nerium (*Nerium oleander)*	Leaf	0.00(1.00)	0.00(1.00)	0.00(1.00)
Neem (*Azadirachta indica)*	Leaf	0.00(1.00)	0.00(1.00)	0.00(1.00)
Adathoda + Soapnut *(A. vessica + S. mukorossi)*	Leaf+ Fruit	1.00(1.41)	1.48(1.57)	1.24(1.50)
Adathoda + Meswak *(A. vessica + S. persica)*	Leaf+ Stem	0.68(1.29)	0.98(1.41)	0.83(1.35)
Meswak + Soapnut (*S. persica + S. mukorossi)*	Stem+Fruit	0.83(1.35)	1.18(1.47)	1.00(1.41)
Mean		0.49(1.22)	0.73(1.32)	0.61(1.27)
Source	SEM±	CD at 1%		
Plant extracts (P)	0.008	0.030		
Concentration (C)	0.004	0.014		
Interaction (P × C)	0.012	0.043		

* √×+1 transformed values

Manjula (2002) observed the significant difference in the inhibitory effect among the eight plant extracts screened against the growth of *X. a.* pv. *punicae*. Kalangi extract (1:1) was found more effective against the growth of Bangalore fruit isolate followed by meswak, tulsi and patchouli, where as meswak exhibited highest inhibitory effect followed by kalangi and patchouli on the growth of Bijapur isolate. Later Jalaraddi (2006) found that both aqueous and alcoholic extracts of garlic, meswak and citronella were inhibitory to growth of the *Xanthomonas axonopodis* pv.*punicae*. Similarly Yenjerappa (2009) reported that among the different plant extracts tested against *X. a.* pv. *punicae*, garlic extract (10%) produced the maximum inhibition zone followed by parthenium, lantana leaf extract and onion bulb extract each at 10 per cent concentration in *in vitro* condition.

In vitro* evaluation of antagonistic bacteria on the growth of *Xanthomonas axonopodis* pv. *punicae

Among the five different strains of *Pseudomonas fluorescens* tested strain no. 326(4) (3.10cm) and 139 (3.00cm) has recorded highest inhibitory zone and these are significantly superior over other strains tested and there is no significant difference between these two strains.. Followed by strain pf1 (2.77cm), 134 (2.72cm) and pf5 (2.65cm) (Table 2).

The antagonism of *Pseudomonas fluorescens* against some *Xanthomonas* spp. was reported by Unnamalai and Gnanamanickam (1984), Sakthivel *et al.*(1986), Safiyazov *et al*. (1995). Jones *et al.* (2011a) reported that, out of 62 fluorescent pseudomonads evaluated, following the dual culture assay, 22 isolates exhibited antagonistic activity against *Xanthomonas axonopodis* pv. *punicae* with inhibition zone varied from 2.0 to 5.0mm.

Among the eleven different strains of PPFM tested, strain 10L has recorded highest inhibitory zone of 0.95cm which is significantly superior over rest of the strains followed by 75L (0.80cm). Other strains were failed to show any inhibitory zone (Table 3).

Jones *et al.* (2011b) reported that, Pink Pigmented Facultative Methylobacterium (PPFM) H5 strain, isolated from Bagalkot, has shown maximum inhibition zone (15.00mm) followed by PPFM 42U (14.60mm), PPFM 71 (14.40mm) and PPFM 75L (14.00mm) against *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate.

In vitro* evaluation of micronutrients on the growth of *Xanthomonas axonopodis* pv. *punicae

Among the nine micronutrients tested, $CuSO_4$ has recorded highest mean inhibitory zone

of 0.89cm which is significantly superior over other strains tested followed by micron special (boron, $MgSO_4$ and $ZnSO_4$) 0.41cm at all concentrations tested. $MnSO_4$, $CaSO_4$, boron, sulfur, $FeSO_4$ were

Table 2. *In vitro* evaluation of *Pseudomonas fluorescens* against *Xanthomonas axonopodis* pv. *punicae*

Pseudomonas fluorescens strains	Inhibition zone (mean diameter in cm)
134	2.72(1.93)*
PF-5	2.65(1.91)
326(4)	3.10(2.02)
PF-1	3.00(2.00)
139	2.77(1.94)
Mean	2.37(1.80)
Sem±	0.03
CD	0.13

* √×+1 transformed values

Table 3. *In vitro* evaluation of PPFM (pink pigmented facultative methylotrophs) against *Xanthomonas axonopodis* pv. *punicae*

Strains	Inhibition zone (mean diameter in cm)
20A	0.00(1.00)*
71LIN	0.00(1.00)
32	0.00(1.00)
10L	0.95(1.40)
75L	0.80(1.34)
42U	0.00(1.00)
23A	0.00(1.00)
80L	0.00(1.00)
26U	0.00(1.00)
38U	0.00(1.00)
H5	0.00(1.00)
CONTROL	0.00(1.00)
Mean	0.15(1.06)
SEM±	0.006153
CD at 1%	0.02

* √×+1 transformed values

Table 4. *In vitro* evaluation of micronutrients against *Xanthomonas axonopodis* pv. *punicae*

Treatment	Inhibition zone (cm) Concentration (%)				
	0.05	0.1	0.25	0.5	Mean
	0.00	0.00	0.00	0.63	0.16
$ZnSO_4$	(1.00)*	(1.00)	(1.00)	(1.27)	(1.07)
$CuSO_4$	0.60	0.75	0.95	1.25	0.89
	(1.26)	(1.32)	(1.40)	(1.50)	(1.37)
$MnSO_4$	0.00	0.00	0.00	0.00	0.00
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
$MgSO_4$	0.00	0.00	0.00	0.60	0.15
	(1.00)	(1.00)	(1.00)	(1.26)	(1.07)
$CaSO_4$	0.00	0.00	0.00	0.00	0.00
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
BORON	0.00	0.00	0.00	0.00	0.00
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
SULFUR	0.00	0.00	0.00	0.00	0.00
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
$FeSO_4$	0.00	0.00	0.00	0.00	0.00
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
MICRON SPECIAL	0.00	0.00	0.65	1.00	0.41
(boron, ZnSO4, FeSO4)	(1.00)	(1.00)	(1.28)	(1.41)	(1.17)
	0.00	0.00	0.00	0.00	0.00
Control	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
	0.06	0.1325	0.21	0.395	0.161
Mean	(1.03)	(1.05)	(1.09)	(1.17)	(1.07)
Sources	Treatment	Concentration		TXC	
SEM	0.002	0.001	0.004		
CD	0.010	0.010	0.020		

* √×+1 transformed values

failed to inhibit the pathogen. $CuSO_4$ has recorded maximum inhibition zone of 0.60cm at 0.05%, 0.75cm at 0.1% and 0.95cm at 0.2% and 1.25cm at 0.5% concentrations respectively (Table 4).

Strong correlation between boron uptake in leaf tissues, residual boron in soil with black rot incidence in cauliflower was reported by Kumar and Kotur (1989). Exogenous supply of boron (0 – 6.4 mg/kg) in low boron containing altisols (hot water soluble soil containing boron of 0.1 mg/kg) indicated that, this micronutrient has a definite role in susceptibility of cauliflower to black rot caused by *Xanthomonas campestris* pv. *campestris*. Susceptibility was greatly observed in boron deficient (below 0.4 mg/kg) and boron excess (above 1.6 mg/kg) plants than the plants grown with optimum level of boron (0.4 – 1.6 mg/kg).

Dordas reviewed the effect of nutrients, such as N, K, P, Mn, Zn, B, Cl and Si, on disease resistance and tolerance. Among the micronutrients, Mn can control a number of diseases, as Mn has an important role in lignin biosynthesis, phenol biosynthesis, photosynthesis. Zn showed decreased or increased susceptibility to disease in some cases. B was found to reduce the severity of many diseases because it has function on cell wall structure, plant membranes and plant metabolism. Nutrients can reduce disease to an acceptable level, or at least to a level at which further control by other cultural practices or conventional organic biocides are more successful and less expensive (Dordas, 2008).

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