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In vitro Evaluation of Antibacterial Activity of Some Plant Leaf Extracts against *Xanthomonas axonopodis* pv. *phaseoli* Isolated from Seeds of Lentil (*Lens culinaris* Medik.)

Kulshrestha S., Chaturvedi S., Jangir R. and Agrawal K.
Department of Botany, University of Rajasthan, Jaipur- 302004, Rajasthan, INDIA

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Abstract

Antibacterial activity of some plant extracts against Xanthomonas axonopodis pv. phaseoli (Xap) was evaluated in vitro. Xap is a Gram negative seed borne pathogen. It was found associated with seeds of lentil and caused bacterial leaf spot disease. Six plant leaf extracts (aqueous and methanolic) were evaluated in different concentrations for their antibacterial activity against Xap through disc diffusion method. Mint (Mentha piperita), black plum (Syzygium cumini) and datura (Datura metel) gave inhibition activity in both aqueous and methanolic extracts while winter cherry (Withania sominifera) showed inhibition only by methanolic extract. Three plant extracts of mint, black plum and datura also improved seed germination and controlled the pathogen on seed treatment significantly at 1% (p≥0.01 %). Highest activity was observed in methanolic extract of mint at a concentration of 250 mg/ml showing mean inhibition zone of 12.1mm and 12.5 mm with IA value of 440.1 mm^2 and 471.0 mm^2 respectively.

Keywords: Antibacterial activity, leaf extracts, disc diffusion method, seed treatment method.

Introduction

Xanthomonas axonopodis pv. *phaseoli* (E. F. Smith) Dowson is a seed borne bacterial pathogen that causes bacterial leaf spot in lentil (*Lens culinaris* Medik.)[1-3] and common bacterial blight in beans. It is Gram-negative aerobic rods belonging to the family *Pseudomonodaceae*. The pathogen causes disease actively at high temperature (up to 32°C) and high rainfall and humidity. The pathogen causes destruction of seedlings and adult plants and thus results in yield reduction[4]. For control of pathogen, pathogen-free seeds and rotation and ploughing of infested straw can be used[5,6].

Use of resistant cultivars, sprays and dust can also control the pathogen but it is controlled normally through chemical bactericides[7].

In recent years, consumers have become more concerned about the safety of food since high use of chemical fungicides, insecticides and bactericides for the control of various plant diseases. These also cause water and soil pollution. They are costly as well as cause health hazards. So it is imperative to use the cost effective and eco-friendly ways to control the diseases through botanicals having antibacterial properties against plant pathogens.

In the present study, leaves of six plant species were used for *in vitro* evaluation of antibacterial activity against *Xap* isolated from seeds of lentil. The leaf extracts were also tested for their effect on germination of lentil seeds.

Material and Methods

X. axonopodis pv. *phaeoli* was isolated from naturally infected seeds of lentil and identified through various biochemical tests and molecular assay. Leaves of Mint (*Mentha piperita),* black plum *(Syzygium cumini),* datura *(Datura metel),* spinach *(Spinacia oleracea),* winter cherry *(Withania sominifera)* and periwinkle *(Catharanthus roseus)* were used for preparation of aqueous and methanolic extracts and their efficacy was evaluated against *Xap* isolated from lentil seeds.

Aqueous extracts were prepared by macerating surface sterilized fresh plant leaves in sterile distilled water in 1:1 w/v. By using double layered sterilized cheese cloth, the extract was filtered and this filtrate was used as 100% concentration. The extract was filtered through double layered sterilized cheese cloth and this filtrate was used as 100% concentration. The extract was further diluted to 50% and 25% concentration with sterilized distilled water. Methanolic extracts were prepared by cold extraction method. Ten gram of powdered leaf was dispersed in 100 ml methanol solution (95%) and kept for 24 to 48 hrs in an incubator shaker at 200 rpm. The obtained suspension was filtered with Whatman filter paper no.1.

Methanol was evaporated from it. The dried extract was stored under dry condition at 4º C[8]. Methanolic extract was used at a concentration of 100 mg/ml, 150 mg/ml and 250 mg/ml.

In vitro evaluation of plant extracts was done by two methods 1 Disc diffusion method and 2 Seed treatment method.

Disc diffusion method: Two isolates of *Xap* (Ac. nos. LC-XAP-5 and LC-XAP- 17) were used and 100µl of pathogen was spread on nutrient agar plates and sterilized discs (5 mm) of Whatman filter paper pre-soaked in extract were further placed on these agar plates. For check discs soaked in distilled water (for aqueous extract) and methanol (for methanolic extracts) were used and placed in the centre of NA plates.

The incubation temperature was 30±2°C applied for 3 to 5 days. Clearance of bacterial culture or zone of inhibition around the discs was recorded. Inhibition annulus (mm^2) was calculated for the comparison of antibacterial activity of the test plant extracts[9,10].

Inhibition Annulus (IA) = $\pi (R_1-R_2) (R_1+R_2)$

Where R_1 = Radius of inhibition zone + radius of filter paper disc, R_2 = Radius of filter paper disc and $\pi = 3.14$

Seed treatment method: Two seed samples (Ac. nos. LC- 102 and LC- 117) naturally infected with *Xap* were used and 3 replicates of 100 seeds per sample were soaked for 1 hr in aqueous and methanolic extracts. Seeds soaked in distilled water, served as check. The seeds were incubated on moistened blotter papers for 7 days to evaluate the effect of leaf extracts on germination of lentil seeds[11]. Percent germination of seeds and percent control of pathogen was calculated.

$$\text{Percent control} = \frac{C - T}{C} X100$$

Where: C= Incidence in Check, T = Incidence in Treated Seeds

Results and Discussion

In disc diffusion method, results were measured in the form of inhibition annulus (IA) values (mm^2) and zone of inhibition (mm). Two isolates of *Xap* were used in disc diffusion method. Spinach and periwinkle did not show any inhibition zone against *Xap* for both the isolates. Mint, black plum and datura gave inhibition zone in both methanolic and aqueous extracts while winter cherry showed inhibition zone only in methanolic extract.

Highest activity was observed in methanolic extract of mint at a concentration of 250 mg/ml (inhibition zone of 12.1mm and 12.5 mm with IA value of 440.1 mm^2 and 471.0 mm^2 respectively) (figure-1D and table-1) and lowest activity was observed in aqueous extract of datura (4.8 mm and 4.5 zone of inhibition with IA value of 52.7 mm^2 and 43.9 mm^2 at maximum concentration of 100%).

Aqueous extract of mint showed inhibition zone of 7.3mm and 8.2 mm with IA value of 147.7 mm^2 and 191.5 mm^2 at 100% concentration. Result showed significance at 1% ($p \geq 0.01$) (figure-1A and table-1). Germination percent was observed highest by the aqueous extract of mint (98.3%) while lowest germination was observed by the aqueous extract of datura (85.3%). Extracts of datura showed variation in seed germination at different concentrations. Percent control of pathogen was highest by methanolic extract of datura in both the samples (69.7 and 69.4% respectively) among three extracts (table-2).

The above mentioned plants were selected because of their different medicinal properties, antifungal effect and herbicidal activities.

Leaves of *Mentha piperita* contains many active compounds such as menthol, menthone, methyl acetate, menthofuran and limnone etc., due to which it shows a significant antibacterial activity[12,13]. Ethanolic extract of black plum was found to possess high mean total activity against *X. campestris* and ethanolic extract of its seeds significantly reduced the ability of the pathogen to retrieve growth on extract free nutrient medium which can be described as post extract effect (PEE)[14].

Kagale et al.[15] have tested the antibacterial activity of *D. metel* leaf extracts against *R. solani* and *X. oryzae* and observed that 90% of colonies were inhibited in medium amended with *D. metel* extract when compared to the control.

Ranaware et al.[16] screened seven plant species against *Alternaria carthami*, among these *Allium sativum, Datura metel* and *Ocimum sanctum* were found more inhibitory activity against the pathogen. Shahnaz et al.[17] reported the inhibitory effect of *Datura alba* against *Macrophomina phaseolina* and *Rhizoctonia solani.* Besides the well known antibacterial effect of these plants, it is necessary to assess their activity against the pathogen and find their germination efficiency after treatment for the sake of crop surveillance and disease prevention both. It is probably because inhibitory activities of plants vary for pathogens and varying effect on germination as it was observed that aqueous extract of seeds and leaves of *D. stramonium* had concentration dependent effect on tested species *i.e.* as the concentration of seed and leaf extracts increased from 0% to 100% its inhibitory effects also increased on tested species[18]. Shafique et al.[19] observed that aqueous treatment of black plum for 10 min generally enhanced germination of wheat grains as compared to control. By contrast, 20 min. treatment of *S. cumini* extracts reduced the germination by 24%.

The inhibitory effects on all tested species increased as the concentration of both extracts increased from 0% to 100%[18]. Shafique et al.[19] observed that aqueous treatment of black plum for 10 min generally enhanced germination of wheat grains as compared to control. By contrast, 20 min. treatment of *S. cumini* extracts reduced the germination by 24%.

Table-1
***In vitro* evaluation of different leaf extracts for inhibition of *X. axonopodis* pv. *phaseoli* by filter paper disc method**

Isolate no. LC-XAP- 5												
Plant Names	**Aqueous extract**						**Methanolic extract**					
	25%		**50%**		**100%**		**100mg**		**150mg**		**250mg**	
	R_{IZ}	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**
Mentha arvensis	5.2	65.2**	6.8	125.5**	7.3	147.7**	11.3	381.3**	11.4	388.4**	12.1	440.1**
Syzygium cumini	3.1	10.5*	3.9	28.1**	5.7	82.3**	5.2	65.2**	7.2	143.1**	11.9	425.0**
Datura metel	3.2	12.5*	3.7	23.3**	4.8	52.7*	6.1	97.2**	6.7	121.3**	8.2	191.5**
Withania sominifera	-	-	-	-	-	-	5.5	75.4**	5.8	86.0**	6.2	101.1**
S.Em.		2.92		3.15		10.03		6.59		3.02		8.66
CD at 5%		11.47		12.39		39.45		22.83		10.46		30
CD at 1%		18.97		20.5		65.27		34.57		15.83		45.42
Isolate no. LC-XAP- 17												
Plant Names	**Aqueous Extract**						**Methanolic Extract**					
	25%		**50%**		**100%**		**100mg/ml**		**150mg/ml**		**250mg/ml**	
	R_{IZ}	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**	**R_{IZ}**	**IA**
Mentha arvensis	5.0	58.9**	6.3	106.3**	8.2	191.5**	11.4	388.5**	11.6	402.9**	12.5	471.0**
Syzygium cumini	2.9	6.8 ^{NS}	4.0	30.6*	5.7	82.4**	5.6	78.8**	7.8	171.4**	12.2	447.7**
Datura metel	2.9	6.8 ^{NS}	3.0	8.6^{NS}	4.5	43.9*	6.1	97.2**	6.2	101.1**	7.7	166.5**
Withania sominifera	-	-	-	-	-	-	5.2	65.3**	5.6	78.8**	5.7	82.4**
S.Em.		4.96		6.52		8.39		5.76		9.66		15.63
CD at 5%		19.48		25.64		32.97		19.94		33.45		54.15
CD at 1%		32.23		42.42		54.56		30.19		50.65		81.99

Values are the mean of three replicates; R_{IZ} – radius of inhibition zone (mm); IA- inhibition annulus (mm^2); **Significant at 1%; *significant at 5%; ^{NS}- Non significant

Table- 2
Percent control of *X. axonopodis* pv. *phaseoli* by leaf extract of different botanicals in seeds of lentil

Plant extracts	Conc. (%)	Seed sample - Ac. No. LC- 102						
		Aqueous extract			Methanolic extract			
		Seed Germination (%)**	Incidence of Pathogen (%)**	Control of Pathogen (%)**	Conc. in mg/ml	Seed Germination (%)**	Incidence of Pathogen (%)**	Control of Pathogen (%)**
Check		78.7(62.51)	36.7 (37.29)	0 (0.00)		75 (60.00)	37.3 (37.64)	0 (0.00)
Mentha arvensis	25	85.3 (67.45)	28.3 (32.14)	22.7 (28.45)	100	81.7 (64.67)	21.3 (27.49)	42.9 (40.92)
	50	92.7 (74.32)	20.3 (26.78)	44.6 (41.90)	150	84.0 (66.42)	18.7 (25.62)	50.0 (45.00)
	100	97.3 (80.54)	18.7 (25.62)	49.1 (44.48)	250	89.7 (71.28)	15.3 (23.03)	58.9 (50.13)
Syzygium cumini	25	87.0 (68.87)	20.3 (26.78)	44.6 (41.90)	100	85.3 (67.45)	22.3 (28.18)	40.2 (39.35)
	50	89.3 (70.91)	15.3 (23.03)	58.2 (49.72)	150	87.7 (69.47)	17.3 (24.58)	53.6 (47.06)
	100	93.7 (75.46)	12.7 (20.88)	65.5 (54.03)	250	90.3 (71.85)	11.3 (19.64)	69.7 (56.60)
Datura metel	25	91.3 (72.84)	21.7 (27.76)	40.9 (39.76)	100	92.0 (73.57)	22.3 (28.18)	40.2 (39.35)
	50	95.0 (77.08)	17.3 (24.58)	52.7 (46.55)	150	89.3 (70.91)	15.7 (23.34)	58.0 (49.60)
	100	92.7 (74.32)	12.7 (20.88)	65.5 (54.03)	250	91.7 (73.26)	11.3 (19.64)	69.7 (56.60)
S.Em.		0.37	0.32	0.36		0.3	0.23	0.29
CD at 5 %		1.1	0.96	1.06		0.89	0.67	0.87
CD at 1 %		1.5	1.31	1.45		1.22	0.92	1.2
Plant extracts	Conc. (%)	Seed sample - Ac. No. LC- 117						
		Aqueous extract			Methanolic extract			
		Seed Germination (%)**	Incidence of Pathogen (%)**	Control of Pathogen (%)**	Conc. in mg/ml	Seed Germination (%)**	Incidence of Pathogen (%)**	Control of Pathogen (%)**
Check		73.0 (58.69)	38.7 (38.47)	0 (0.00)		76.7 (61.14)	40.3 (39.41)	0 (0.00)
Mentha arvensis	25	90.3 (71.85)	26.7 (31.11)	31.0 (33.83)	100	85.3 (67.45)	22.3 (28.18)	44.6 (41.96)
	50	95.7 (78.03)	22.7 (28.45)	41.4 (40.05)	150	87.7 (69.47)	19.3 (26.06)	52.1 (46.20)
	100	98.3 (82.51)	15.3 (23.03)	60.4 (51.00)	250	92.7 (74.32)	12.7 (20.88)	68.6 (54.09)
Syzygium cumini	25	87.7 (69.47)	21.7 (27.76)	44.0 (41.55)	100	82.3 (65.12)	22.7 (28.45)	43.8 (41.44)
	50	91.3 (72.84)	20.7 (27.06)	46.6 (43.05)	150	89.0 (70.63)	18.7 (25.62)	53.7 (47.12)
	100	95.7 (78.03)	18.3 (25.33)	52.6 (46.49)	250	93.7 (75.60)	16.0 (23.58)	60.3 (50.94)
Datura metel	25	86.0 (68.03)	21.3 (27.49)	44.8 (42.02)	100	90.0 (71.56)	19.7 (26.35)	51.2 (45.69)
	50	89.7 (71.28)	17.7 (24.88)	54.3 (47.47)	150	91.3 (72.84)	14.7 (22.55)	63.6 (53.89)
	100	85.3 (67.45)	14.3 (22.22)	62.9 (52.48)	250	87.3 (69.12)	12.3 (20.53)	69.4 (56.42)
S.Em.		0.59	0.38	0.26		0.15	0.27	0.32
CD at 5 %		1.76	1.14	0.79		0.46	0.8	0.96
CD at 1 %		2.41	1.56	1.08		0.63	1.09	1.32

Values are the mean of 3 replicates; values in parenthesis are angular transformed values; ** Significant at 1%

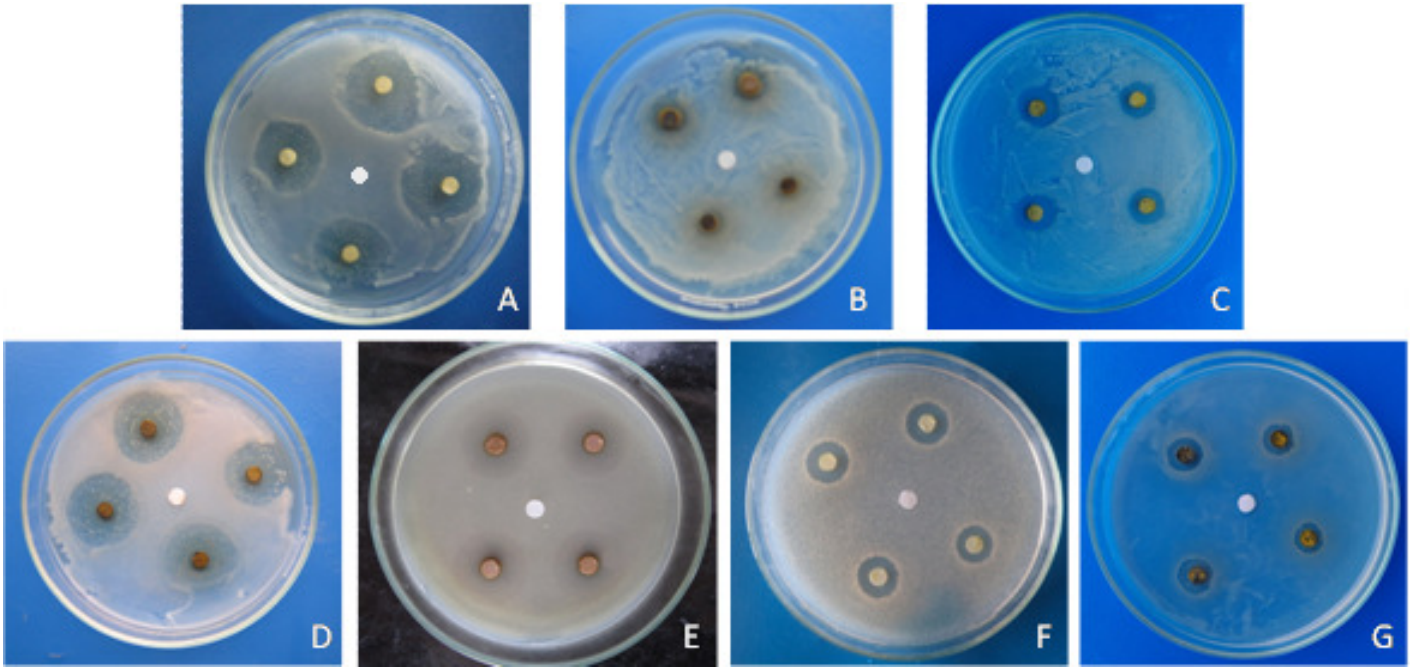

Figure-1

***In vitro* evaluation of aqueous (100% concentration) and methanolic extracts (250mg/ml concentration) through disc diffusion method. A-C: Aqueous extract of Mint (A), Black plum (B) and Datura (C); D-G: Methanolic extract of Mint (D), Black plum (E), Datura (F) and Winter cherry (G); Check is placed in centre of Petri plate**

Conclusion

Thus in present study mint, black plum and datura gave inhibition activity in both aqueous and methanolic extracts while winter cherry showed inhibition only by methanolic extract against *Xanthomonas axonopodis* pv. *phaseoli* isolated from seeds of lentil. No activity was found in extracts of spinach and periwinkle. Three plant extracts mint, black plum and datura also improved seed germination and controlled the pathogen on seed treatment significantly at 1% ($p \geq 0.01$ %).

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