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Management of bacterial blight of cotton (*Xanthomonas campestris* pv. *malvacearum*) through plant extracts and its impact on yield components of bt cotton varieties

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Abstract

Cotton is known as the king of fibers. Bacterial blight of cotton is a devastating cotton disease of subtropical and tropical regions. Five plant extracts *Azadirachta indica*, *Moringa oleifera, Cassia fistula, Allium cepa* and *Eucalyptus oblique* were evaluated at 5%, 10% and 15% concentrations against colony growth of *Xanthomonas campestris pv. malvacearum* using inhibition zone technique. At 15% concentration, *Azadirachta indica* was most effect to inhibit bacterial colony with respect to others. Plants extracts having maximum inhibitory effect against the pathogen were evaluated on four different Bt-cotton varieties in field conditions against the disease and biological parameters. Comparing to the extracts applied, *Azadirachta indica* considerably reduced the disease incidence on all experimental varieties (Bt-866, Bt-113, Bt-92 and Bt-802). Plants of 802 variety sprayed with Neem extract were more in height (93.8 cm), number of bolls (40) and leaves (106) as compared to the other plants of different varieties where other extracts applied. The extract of *Azadirachta indica* at 15% concentration can be used as alternative to antibiotics to manage the bacterial blight disease of cotton.

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Introduction

Cotton is a widely sown cash crop in tropical and sub-tropical regions of the world. It is unique among fibrous crops known as white gold. Taxonomically, cotton belongs to family "Malvaceae" and genus "Gossypium" having thirty-five species. Only four species (*G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. herbaceum*) are commercially cultivated (Hutchinson, 1947) in the world due to physico-chemical properties of their fibers. In 2014 Cotton was cultivated in the area of 35.7 million hectares with annual production 123.3 million bales (Johnson *et al*., 2012). Pakistan is the 4th major cotton producing country in the word, more than 3350 thousand hectares were sown with 14 million bales production in 2014-15. Plant diseases debase about 220$ millions annually around the globe, the losses are more in developing countries where resources and facilities are limited (Agrios, 2005).

Cotton is vulnerable to many diseases which significantly lowers the yield every year (Hillocks, 1992b). Bacterial Blight of cotton is a bacterial disease caused by *Xanthomonas campestris* pv. *malvacearum* (Hillocks, 1992a). Disease losses varies from region to region. In Asian and African fields, losses are not unusual from 10-30 percent (Kirkpatrick and Rockroth, 2001). However, more than 50% losses were recorded in severe conditions (Verma, 1986). The disease starts primarily from infected seed, plant may die at seedling stage. On leaves, angular water soaked spots appear firstly on the lower surface then at the both sides. With the passage of time, these spots becomes more conspicuous and brownish in color. In severe conditions, the spots coalesces and gives blighted symptom. On stem, the corky spots favors the breakage when air blows, termed as "black arm". From the corky spots on bolls, bacterium travels to the seed through the lint and serves as a pathogen's reservoir for next infection (Singh, 2009).

Indeed pesticides have saved the millions of dollars but their alarming use has defiled our environment as well. The dangerous elements has entered in our food web and denaturing the life of many species with the passage of time. Plants extracts have been reported to have pesticidal properties (Hammer *et al*., 1999; Satish *et al*., 1999), this could be hope to save our environment from antibacterial agrochemicals. The revolution of Genetically Modified Crops has opened the new era in science (Khush, 2001). Insertion of Bt-genes in cotton germplasm has contributed much to lowers the insecticide usage (Zhang *et al*., 2000).

Keeping in view the above facts, present study was designed to evaluate the efficacy of different plant extracts against the *Xanthomonas campestris* pv. *malvacearum* causing bacterial blight of cotton diseases in lab conditions. In field conditions, impact of plant extracts will be assessed on Bt-cotton varieties by measuring plant height, number of leaves and bolls per plant against bacterial blight of cotton disease.

Materials and methods

Isolation, purification and multiplication of the pathogen

Diseased symptomatic leaves from infected cotton plants were taken from experimental area of Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. and were preserved at 4 ^{o}C for further study. Diseased tissues were excised and surface sterilized in 0.1% $HgCl_2$ for two minutes then rinsed twicely with distilled water and air dried. Disease tissues were macerated in sterilized water and resultant was filtered by Watman No.1 filter paper. Three successive dilutions (10^{-1}, 10^{-2} and 10^{-3}) were made and bacterium was isolated by dilution plate technique. Modified Nutrient Glucose medium (glucose= 5g/L and nutrient agar = 15g/L) was used for bacterial colonies isolation. Plates were wrapped and incubated for 48 hours at 25 ^{o}C. Recovered colonies were purified by streaking method and preserved (50% glycerin solution) for further study at 4 ^{o}C.

Preparation of the aqueous plant extracts

Leaves of each plants were taken, thoroughly washed with tap water and dried in the sun. Giving brittle

appearance, leaves were grinded by electric grinder. Twenty five gram powder of each extract was taken dissolves in 100 ml of acetone solvent. Extracts were mixed thoroughly by electric stirrer. The aliquot was poured in to plastic tubes and centrifuged at 6000rpm for 5 minutes. After centrifugation, extract was taken out by the help of pippet and solution was passed through Whatman No.1 filter paper. The resultant was considered to be standard and further 5%, 10% and 15% dilutions were made.

In vitro evaluation of plant extracts against bacterial colony growth

Freshly growing aqueous bacterial culture was prepared in nutrient broth which was mixed in sterilized Nutrient Glucose agar medium and then was poured in Petri plates. After solidification, wells of 1 cm were made at center by sterilized cork borer and extracts were poured by sterilized pippet. In control, sterile water was poured. Treated plates were wrapped and incubated at 25 ^{o}C. Data was recorded by measuring the inhibition zones area with an ordinary ruler in centimeter scale after 72 hours. Experiment was conducted in Completely Randomized Design (CRD) with three replications.

Plant extracts efficacy against bacterial of cotton disease in field conditions

Seeds of four cotton varieties (Bt-866, Bt-113, Bt-92 and Bt-802) were taken from Cotton Research Station, Ayub Agricultural Research Institute, Faisalabad, Pakistan. Prior to sowing, linted seeds were immersed in bacterial suspension for two hours to maximize the seed born infection chance. Soaked seeds were sown in experimental area of Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Randomized Complete Block Design was adopted with three repeats. All agronomic practices were followed. On three weeks old plants, bacterial suspension (1 × 108 CFU/ml) was sprayed twice with four days interval on four cotton varieties (Bt-866, Bt-113, Bt-92 and Bt-802) for the disease infection. Extracts of *Azadirachta indica*, *Eucalyptus camaldulensis* and *Moringa oleifera* exhibited significant antibacterial efficacy against *Xanthomonas campestris* pv. *malvacearum* in lab condition were sprayed at 15% concentration on six weeks old diseased cotton plants. Nothing was applied in control treatment. Plant height, bolls and leaves per plant were recorded after three weeks of extract's application.

Statistical analysis

Recorded data was analyzed through Analysis of Variance (ANOVA) and treatments means were compared by Fisher's Least Significant Difference (LSD) test. Data was processed statistically through SAS (9.3) software (Inc., 2011-2012) and was represented by Microsoft Excel (2013) (Wilson, 2014).

Results

In vitro efficacy of different plant extracts against bacterial blight of cotton pathogen

Plant extracts efficacy against *Xanthomonas campestris* pv. *malvacearum* varied significantly at the different concentrations in lab conditions (Table.1).

Table 1. *In vitro* efficacy of plant extracts at different concentration on *Xanthomonas campestris pv. malvacearum* colony growth (ANOVA).

Source	DF	SS	MS	F	P
Treatments	5	4.29068	0.85814	346.51	0.0000**
Dose	2	3.51175	1.75587	709.00	0.0000**
Treatments x Concentration	10	0.81980	0.08198	33.10	0.0000**
Error	36	0.08916	0.00248		
Total	53	8.71138			

** = Highly Significant

α = 0.05.

After 72 hours of application, at 5% concentration, *Azadirachta indica* was found most significant to inhibit bacterial colony growth (0.53 cm) as compared to other treatments. No significant difference in inhibition zone's diameter was measured among *Cassia fistula*, *Eucalyptus camaldulensis* and *Moringa olifera* extracts. *Allium cipa* was least significant to inhibit bacterial colony of *Xanthomonas campestris* pv. *malvacearum* as compared to other extracts.

Table 2. Impact of plant extracts on disease incidence and yield incidence on different cotton plant at filed conditions (ANOVA).

Source	Comparison	DF	MS	F
Disease Incidence	Treatments x Varieties	9	5.393	341.65*
Plant height	Treatments x Varieties	9	5.449	13.33*
Number of balls	Treatments x Varieties	9	2.389	14.70*
Number of leaves	Treatments x Varieties	9	5.40	18.69*

* = Highly significant

α = 0.05.

With the increase of concentration, extracts efficacy against the bacterial colony increases significantly. At 10 % concentration, extracts of *Cassia fistula, Moringa olifera* and *Allium cipa* were significantly same to inhibit bacterial colony. *Eucalyptus camaldulensis* was found more significant as compare to *Cassia fistula, Moringa olifera* and *Allium cipa* but not from *Azadirachta indica*. *Azadirachta indica* was most effective as compare to other treatments.

Table 3. Relative efficacy of different plant extracts against bacterial blight incidence with respect to different cotton varieties at field conditions.

Treatments	Cotton Varieties			
	BT-866	BT-113	BT-92	BT-802
Azadirachta indica	55.80 F	51.60 J	47.10 M	42.13 N
Eucalyptus camaldulensis	59.767 C	55.13 G	50.06 K	49.33 L
Moringa oleifera	63.333 B	58.73 E	54.00 H	53.17 I
Control	67.167 A	63.41 B	59.37 D	55.24 G
LSD	0.21			

Mean values sharing similar letters do not differ significantly.

α = 0.05.

At 15% concentration, significant increase in inhibition zone area was observed as compare to at 10% concentration. *Azadirachta indica* was found most significant to inhibit *Xanthomonas campestris* pv. *malvacearum* colony of as compare to other treatments. Extract of *Eucalyptus camaldulensis* was least significant comparing to *Azadirachta indica* but was more significant to *Cassia fistula*, *Moringa olifera* and *Allium cipa*. *Moringa olifera* was found more significant with respect to *Allium cipa* and *Cassia fistula*. No significant difference between antibacterial efficacy of *Cassia fistula and Allium cipa* was recorded, these were least significant as compared to others in inhibiting the bacterial growth (Fig 1).

Plant extracts impact on bacterial blight of cotton disease incidence disease in field conditions

Significant impact of plant extracts was seen to reduce the disease incidence on all three cotton

varieties (Table 2). *Azadirachta indica* was found most significant to reduce the disease incidence on all cotton varieties. The plants of Bt-866 in control treatment exhibited the maximum disease incidence as compared to the plants of other varieties where *Azadirachta indica, Eucalyptus camaldulensis* and *Moringa olifera* extracts sprayed. Minimum disease incidence was observed on Bt-92 sprayed plants with *Azadirachta indica*.

Table 4. Efficacy of different plant extracts on plant height (cm) with respect to different cotton varieties against bacterial blight of cotton disease incidence at field conditions.

Treatments	Cotton Varieties			
	BT-866	BT-113	BT-92	BT-802
Azadirachta indica	81.3 C	87.3 B	87.3 B	93.8 A
Eucalyptus camaldulensis	76.4 E	79.4 D	79.0 D	82.0 C
Moringa oleifera	70.7 F	75.7 E	76.1 E	78.8 D
Control	63.4 I	66.0 H	66.7 H	69.6 G
LSD	1.06			

Mean values sharing similar letters do not differ significantly.
α = 0.05.

The efficacy of *Eucalyptus camaldulensis* and *Moringa olifera* was less significant to reduce the disease incidence of bacterial blight of cotton with respect to the *Azadirachta indica*. *Moringa olifera* was found least significant among extracts against the disease progression (Table 3).

Impact of plant extracts on yield components

Plant height

Significant effect on plant height was observed by the plant extracts on four cotton varieties (Table 2). Bt-802 variety was most significant in plant height as compared to other varieties (Bt-866, Bt-113 and Bt-92) where *Azadirachta indica* was applied at 15% concentration. *Azadirachta indica* was found most significant in plant height on all four cotton varieties as compared to other plant extracts. *Eucalyptus camaldulensis* was more significant with respect to *Moringa olifera* and control treatments.

In control, where nothing was applied, minimum height was observed as compared other extract's treated plants. No meter which plant extract applied, the plants of Bt-802 were most significant in height comparing to Bt-866, Bt-113 and Bt-92 varieties. No significant difference in plant height was recorded between Bt-113 and Bt-92 varieties by all plant extracts. Plants of Bt-866 were significantly smaller in height than other varieties (Table 4).

Number of bolls

Significant effect on number of bolls per plant was seen by the all plant extracts on four cotton varieties (Table 2).

On Bt-802, maximum number of boll (40 bolls) were counted than 866, Bt-113and Bt-92 varieties where *Azadirachta indica* was applied in field conditions. No significant difference in number of bolls were recorded from Bt-113 and Bt-92 *Azadirachta indica* treated plants. Bt-866 plants treated with *Azadirachta indica* were least significant in bearing of cotton bolls but were more significant as compared to other extracts treated plants of the same variety.

Eucalyptus camaldulensis was more significant as compare to *Moringa olifera* and Control treatments on all cotton varieties. Number of bolls counted on *Moringa olifera* treated plants were least significant as compare to *Azadirachta indica* and *Eucalyptus camaldulensis* treated plant's bolls. In control, the number of bolls were least significant to other extracts treated plant on all cotton varieties (Bt-866, Bt-113, Bt-866 and Bt-92) (Table 5).

Table 5. Efficacy of different plant extracts on number of bolls with respect to different cotton varieties against bacterial blight of cotton disease incidence at field conditions.

Treatments	Cotton Varieties			
	BT-866	BT-113	BT-92	BT-802
Azadirachta indica	30 C	34 B	34 B	40 A
Eucalyptus camaldulensis	24 F	27 E	29 D	34 B
Moringa oleifera	20 J	22 H	23 G	27 E
Control	18 K	20 J	21 I	24 F
LSD	0.67			

Mean values sharing similar letters do not differ significantly.

α = 0.05.

Number of leaves

Leaves production significantly varied by Plant extracts application with respect to different cotton varieties (Table 2). Maximum number of leaves (106) were seen in Bt-802 plants where *Azadirachta indica* was applied. Bt-92 plants treated with *Azadirachta indica* were least significant in leaves producing as compared to Bt-802. *Azadirachta indica* treated plants but were more significant as compared to other treated plants of the same variety.

The plants of Bt-866, Bt-113, Bt-866 and Bt-92 where *Moringa olifera* was applied were least significant in leave producing as compared to the other plants where the aqueous extracts of *Azadirachta indica* and *Eucalyptus camaldulensis* sprayed. The extract of *Azadirachta indica* was found most effective as compared to others on all cotton plants. In control where nothing was applied, the plant were significantly lower in leaves as compared to those where extracts were applied (Table 6).

Table 6. Impact of plant extracts on number of leaves per plant with respect to different cotton varieties against bacterial blight of cotton disease incidence at field conditions.

Treatments	Cotton Varieties			
	BT-866	BT-113	BT-92	BT-802
Azadirachta indica	92 D	95 C	98 B	106 A
Eucalyptus camaldulensis	80 H	86 F	89 E	94 C
Moringa oleifera	73 K	76 J	78 I	84 G
Control	68 M	71 L	74 K	77 J
LSD	0.9			

Mean values sharing similar letters do not differ significantly.

α = 0.05.

Discussion

Bacterial blight is a devastating cotton disease significantly lowers the yield in epidemic form. Chemicals are authentic way for the disease management but has badly polluted our biotic and a-biotic environment. To evaluate the products against the bacterial blight at field conditions is a time consuming and expensive practice. Inhibition zone technique is an easy and cheap approach to evaluate the therapuents against the pathogen in lab conditions.

Azadirachta indica is commonly known as "Neem" actively contains nimbine and nimbinine in its leaves which disrupts the cell wall by electrolyte's leakage resulting into the cell death. *Moringa olifera* leaves have the rich sources of moringine (benzylamine), moringinine and spirochin compounds, efficiently inhibits the bacterial growth. The leaves of the of *Eucalyptus camaldulensis* having a rich source of ineole, caryophyllene, a-pinene and limonene found efficiently to inhibit the bacterial growth.

Inhibition zone is a cost benefit technique to evaluate the test products against the pathogens, however, it also has some limitations and it directly depends upon the nature of the chemical (viscosity and solubility) and the a-biotic conditions where the experiment is carrying out. Porosity of the ager greatly fluctuates by the temperature, increasing temperature expands the ager's atoms results into decrease of the spaces in the particles and hence, the test chemical's passage reduces considerably. Viscosity is another factor which considerably impacts on this technique, more the test chemical will be viscous, less the liquid passage will occur. Solubility of a particular test chemical directly effects on the technique, the strong antimicrobial colloidal solutions may not be proved effective against a pathogen as the effective particles may not penetrate through the agar pores hence makes small inhibition zone. In field, conditions are totally different from the lab where a-biotic environmental factors fluctuates considerably.

The effectively proved antibacterial compounds in lab conditions may become ineffective in the field due to dissociation by changing environmental factors. Bambawale *et al.* (1995) evaluated ethanol prepared extracts (1:1) of fourteen different plants against bacterial blight of cotton pathogen in lab conditions. Out of 14 *extracts Datura metel and D. stramonium* inhibited significant bacterial activity of the pathogen. Javed *et al.* (2013) assessed four aqueous plant extracts (*Azadirachta indica, Moringa olifera, Datura alba* and *Syzgyium cumini*) at 5%, 10% and 15% concentrations against colony of *Xanthomonas campestris* pv. *malvacearum* using inhibition zone technique. *Datura alba* was the most significant to inhibit bacterial colony (2.1 cm) at 15% concentration. Simmilar studies were carried out by Sajid *et al.* (2013) where three plant extracts (*Citrullus colocynthis, Nicotiana tobaccum and Curcuma Lunga*) were evaluated in vitro by using inhibition zone technique against colony growth of *Xanthomonas axonopodis* pv. *malvacearum*.

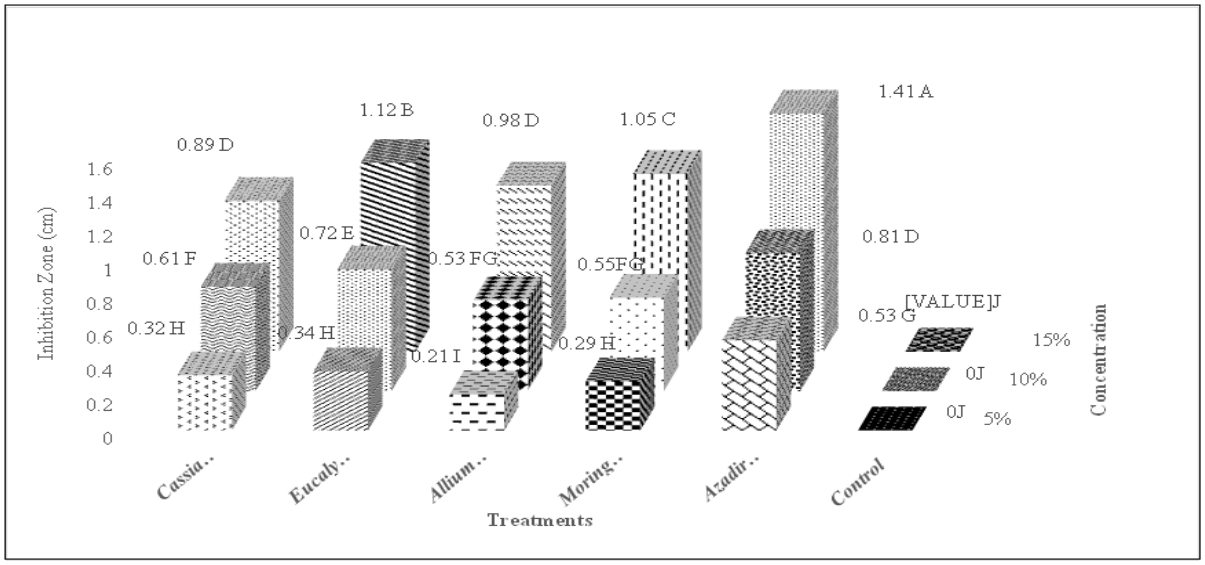

Fig. 1. Efficacy of different plant extracts at different concentrations against colony growth of *Xanthomonas campestris* pv. *malvacearum* using inhibition zone technique.

The source cells in the leaves produce glucose by photosynthesis process (Gifford and Evans, 1981) and transport it to other sink cells in the plant. The glucose is collected by tertiary veins in the leaves and transported in the other vital plant parts where no photosynthesis occurs like stem and roots (Kuzyakov, 2001).

Maximum rate of photosynthesis occurs when all the source cells works efficiently and their products are transported properly through the channels (veins). *Xanthomonas campestris* pv. *malvacearum* is a necrotroph, enters in the leaves through stomata or wounds.

The leaf spotting bacterium secrets the toxins which alters the cell metabolism (Kelman, 1979), disintegrates the plasmallema and degrades the middle lamella of the cell wall (Addy, 1976; Daly, 1976) . The attacked cells lose their viability for photosynthesis process and fails to produce the glucose and its translocation. The optimal pathogen's proliferation conditions increase the necrosis rate which considerably reduces the glucose production results into bad impact on plant's physiology.

The short supply of photosynthates impacts drastically on vegetative and reproductive growth of the plant results into reduced plant height, number of leaves and bolls per plant as compared to the healthy ones. Khan *et al*. (2000) evaluated leaf extracts of Datura alba, seed oil of neem (*Azadirachta indica*), neemseed bitter and nimbokil 60 EC on the of *Xanthomonas campestris* pv. *malvacearum* growth in vitro and in green house conditions. Comparative to untreated control, less number of leaf shedding, less number of bare nodes and more number of boils, increased boil weight and yield of seed cotton of varieties sprayed with standard concentrations of *Datura alba* and nimbokil 60 EC. Raghavendra *et al*. (2007) prepared seaweed (Dravya) extract and evaluated against bacterial bight of cotton by immersing seed before sowing and foliar applications after 10, 20 and 30 days of after sowing. Reduction in disease incidence 66%, 70% and 74% was seen after 40, 60 and 80 days of sowing respectively and increase in plant height, total number of bolls formed, boll weight, stem girth, chlorophyll content, total phenols and peroxidase activity was observed.

Conclusion

The blind use of chemicals has defiled our environment. From our current study it is concluded that the plant extracts have the potential impact against the pathogen's growth. The aqueous extract of *Azadirachta indica* at 15 % concentration significantly hindered bacterial activity in lab and in field conditions can be used potentially to manage the bacterial blight of cotton disease as a chemical's alternative.

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