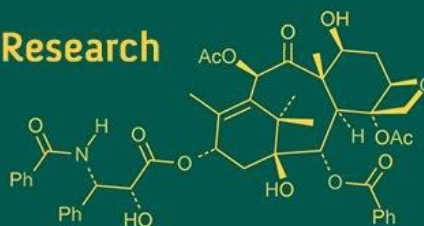


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Evaluation of *Ammannia baccifera* L. against *Fusarium oxysporum* causing wilt disease of chickpea under *in vitro* condition

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Abstract

This research explores the antifungal potential of *Ammannia baccifera* L. against *Fusarium oxysporum* f. sp. *ciceris* (FOC), using *in vitro* methodologies. Initially, aqueous extracts of eight plants viz., *Solanum xanthocarpum*, *Ammannia baccifera*, *Clitoria ternatea*, *Cassia tora*, *Xanthium strumarium*, *Tridax procumbens* and *Achyranthes aspera* were screened using poisoned food technique against FOC. Out of them, the highest percent growth inhibition was observed in case of *A. baccifera* (80.37%), followed by *S. xanthocarpum* (76.30%). Considering this, the crude extract of *A. baccifera* was further evaluated for an *in vitro* seed germination assay at a concentration of 0.5% along with the fungicide Thiophanate methyl (@ 3 g/kg seeds) for comparison. The highest germination (100%) and lowest percent disease incidence (20%) was observed in case of treatment with Thiophanate methyl followed by *A. baccifera* extract where germination percent was recorded as 99.17% and percent disease incidence was found to be 20.83%. both the treatments were statistically at par when compared with the control. These results show that the crude extract of *A. baccifera* can be a promising candidate in the avenue of natural fungicides.

Keywords: Antifungal activity, botanical extracts, *Fusarium*, *Ammannia baccifera*

Introduction

Chickpea (*Cicer arietinum* L.), one of the key legumes cultivated globally, is a popular ingredient in different cuisines. When paired with a cereal based diets, it becomes an essential source of a complete plant-based protein. Additionally, the crop provides ecological services through the mechanism of biological nitrogen fixation due to its symbiotic association with *Rhizobium* bacteria, thus improving soil health and contributing to eco-friendly agricultural practices (Merga and Haji, 2019) [8]. Despite these benefits, its cultivation is hindered by several biotic and abiotic factors. Among these factors, one of the most important issue is the wilt disease caused by *Fusarium oxysporum* f. sp. *ciceris* (FOC), which drastically reduces crop yields (Singh and Vyas, 2021) [10]. The pathogen is capable of persisting in soil and seeds for extended periods by forming chlamydospores. When it infects plants, it penetrates the root epidermis and invades the vascular tissues, reproducing asexually through macroconidia and microconidia. Infected seeds can experience reduced germination, seed rot, or even abortion, while the plants at various growth stages exhibit symptoms like wilting (Jiménez-Díaz *et al.*, 2015) [4]. Splitting the roots of affected plants often reveals dark brown discoloration in the xylem (Nene *et al.*, 1991) [9]. Notably, even seeds that appear normal can harbor the pathogen. FOC is categorized into eight physiological races (0, 1A, 1B/1C, 2, 3, 4, 5, and 6), which induce distinct symptoms. For instance, races 0 and 1B/1C primarily cause yellowing, while races 1A, 2, 3, 4, 5, and 6 are associated with wilting (Jiménez-Gasco and Jiménez-Díaz, 2003; Jiménez-Gasco *et al.*, 2001) [5, 6]. Traditionally, chickpea wilt has been managed through resistant crop varieties and fungicides. However, these strategies face limitations due to the emergence of new pathogen races and potential fungicide resistance, raising concerns about environmental impact and pathogen adaptability (Haware and Nene, 1982) [3]. The use of chemical fungicides to control plant diseases is a popular practice among the farmers because of their

quick results. Indeed, the diseases are controlled, injudicious use of these agro-chemicals have led to various human health issues and environmental hazards.

Many pathogens have developed resistance to certain fungicides. With the adoption of Integrated Plant Disease Management (IPDM), efforts have been diverted towards the utilization of other management strategies but still chemical fungicides are having the lead. In the era of sustainable agriculture, biological control is the most suitable alternative to these chemicals and botanicals are an important part of it. The use of plants and their products in the control of plant diseases was traditionally adopted by the mankind since ages. Use of Neem, Turmeric, Ginger, Garlic, Castor, Dhatura, Marigold, Chrysanthemum, etc. to control crop pests, diseases and nematodes is a very well-known fact. It is an on - going search to discover new plants species to evaluate their antimicrobial activities. The best sources for this are the indigenous and naturalized plants that are utilized in the traditional and folk medicine since years. Naturalized plants are those which were not native to a region but due to their purposeful or accidental introduction, they started growing and became well established there. Most of the plants utilized in this study are naturalized. Plant products are versatile when it comes to their applications in human medicine. Phyto- compounds are extensively used in human medicine since ancient times and are becoming popular in modern times as well due to the development of resistance in microbes to the widely used antibiotics. Hence, plants seem a better alternative for both human and plant health. Till date only a small portion of the plant species from the several hundred thousand species of plants on the globe have been tested for their phytochemical profiling and antimicrobial potencies. Plant products are safe to use because of they being easily biodegradable, thus having no residue problems and proving themselves safe for the environment. So even if, they are having any toxic or antinutritional compounds, they will easily degrade in the sunlight, thus not harming any farm animals. Preparation of an aqueous botanical formulation requires no fancy instruments or sophisticated methodologies. Moreover, they are easy to use in the field and can be adopted effectively with other disease management tactics. Most of the plants are locally available in abundance and thus prove themselves economical. If utilized in a correct manner, phytoextracts can certainly reduce the intensive use of chemical fungicides and would be a great help in reducing the farming costs. Also, they generally pose no threat to beneficial insects and microbes which is a plus point when it comes to the maintenance of the agricultural biodiversity. Considering all these facts, the current study is aimed to determine the antifungal nature of the most effective phyto extract through the means of *in vitro* experiments which forms the basic part of any preliminary screening for antimicrobial compounds.

Materials and Methods

The experiment was conducted during 2020-21 at the Department of Plant Pathology, N. M. College of Agriculture, Navsari Agricultural University, Navsari. The poisoned food technique was designed with 9 treatments and 3 replications whereas the seed germination assay was designed with 3 treatments and 6 replications. Completely Randomized Design (CRD) was followed in both the experiments.

Isolation of the pathogen

Chickpea seeds were used to isolate the seed borne fungi. The surface sterilization was carried out by dipping the seeds in 1% sodium hypochlorite solution followed by three successive washings with sterile distilled water. These seeds were then transferred in Petri plates containing PDA (Potato Dextrose Agar) medium and were incubated at $27 \pm 1^\circ\text{C}$ in BOD incubator for 7 days with 12/12 hour alternating light and dark period. The mycelial growth of the fungus was used for obtaining the pure culture by agar plug method. The pure cultures thus obtained were subjected to microscopic examination along with the colony characters, aiding in identifying the fungi.

Preparation of Crude Extract of the selected Plants

The healthy plant material was collected from and around Navsari Agricultural University. Followed by thorough washing with tap water and distilled water, the plant material was shade dried and ground into a fine powder followed by passing through 1 mm mesh sieve. For preparing the aqueous formulation, the powdered plant material (about 50 g) was soaked in 50 ml of distilled water (1:1 ratio) for 5 hours. The resultant thick paste was filtered through a muslin cloth and the obtained filtrate was centrifuged at 5000 rpm for 10 minutes, followed by filtration through Whatman No. 1 filter paper. The obtained extract was kept as stock solution. The details about the plants and their parts used in this study are as follows: *Solanum xanthocarpum* L. (leaves), *Ammannia baccifera* L. (leaves), *Achyranthes aspera* L. (inflorescences), *Lantana camara* L. (leaves), *Xanthium strumarium* L. (leaves), *Clitoria ternatea* L. (leaves), *Tridax procumbens* L. (leaves) and *Cassia tora* (leaves and pods).

Evaluation of the aqueous formulations against the phytopathogens

10 ml of the aqueous extract was mixed separately in required amount of sterilized PDA medium under aseptic condition. From PDA mixed with extracts, 20 ml was poured into sterilized petri plates. The plates were inoculated with the mycelial disc of the test pathogen. Control was maintained where mycelial disc was placed on the medium without phytoextract. The inoculated plates then were incubated at $27 \pm 1^\circ\text{C}$ in BOD incubator for seven days. The observation on mycelial growth (mm) and percent growth inhibition of test fungi was recorded after 7 days of incubation. The percent growth inhibition (PGI) of pathogen in each treatment was calculated by following formula (Vincent, 1947) ^[11].

$$\text{PGI} = \frac{C - T}{C} \times 100$$

Where,

PGI = Percent Growth Inhibition, C = Colony diameter (mm) in control plate and T = Colony diameter (mm) in treated plate.

To check the efficacy of the most effective phytoextract on seed germination and seed protection.

The aqueous extract of 0.5% concentration was prepared. 60 healthy and surface sterilized seeds were soaked in the fungal suspension (1×10^4 spores/ml) for 30 minutes. Out of

these, 20 seeds were soaked in the plant extract for 1 hour and 20 seeds were treated with the fungicide. The seeds of all the sets were allowed to germinate on three layers of pre sterilized moistened blotter paper and were incubated at room temperature for five days. The germination percentage, percent disease incidence and percent disease control were calculated as follows:

$$\text{Germination Percentage} = \frac{\text{Total no. of seeds germinated}}{\text{Total no. of seeds observed}} \times 100$$

$$\text{PDI} = \frac{\text{Total no. of infected seeds}}{\text{Total no. of seeds observed}} \times 100$$

$$\text{PDC} = \frac{\text{PDI in Control} - \text{PDI in treatment}}{\text{PDI in control}} \times 100$$

Where,

PDI = Percent Disease Incidence

PDC = Percent Disease Control

Results and Discussion

Evaluation of the aqueous formulations of plants against FOC

The significantly highest percent growth inhibition was

observed in the case of *A. baccifera* (80.37%) followed by *S. xanthocarpum* (76.30%). The other extracts that inhibited more than 50% mycelial growth of FOC were *X. strumarium* (68.14%) and *C. tora* (53.33%). The remaining other extracts viz., *L. camara* (40.37%), *A. aspera* (26.30%), *T. procumbens* (25.92%) and *C. ternatea* (19.26%) were not much effective against FOC. The observations are presented in Table 1 and Figure 1.

Table 1: Evaluation of aqueous formulations of plants against FOC

Sr. No.	Treatment	Average colony diameter (mm)	Percent growth inhibition
T ₁	<i>S. xanthocarpum</i>	21.33	76.30
T ₂	<i>A. baccifera</i>	17.67	80.37
T ₃	<i>A. aspera</i>	66.33	26.30
T ₄	<i>L. camara</i>	53.67	40.37
T ₅	<i>X. strumarium</i>	28.67	68.14
T ₆	<i>C. ternatea</i>	72.67	19.26
T ₇	<i>T. procumbens</i>	66.67	25.92
T ₈	<i>C. tora</i>	42.00	53.33
T ₉	Control	90.00	-
S.E.m. ±		1.05	
C.D. at 5%		3.15	
C.V. %		3.6	

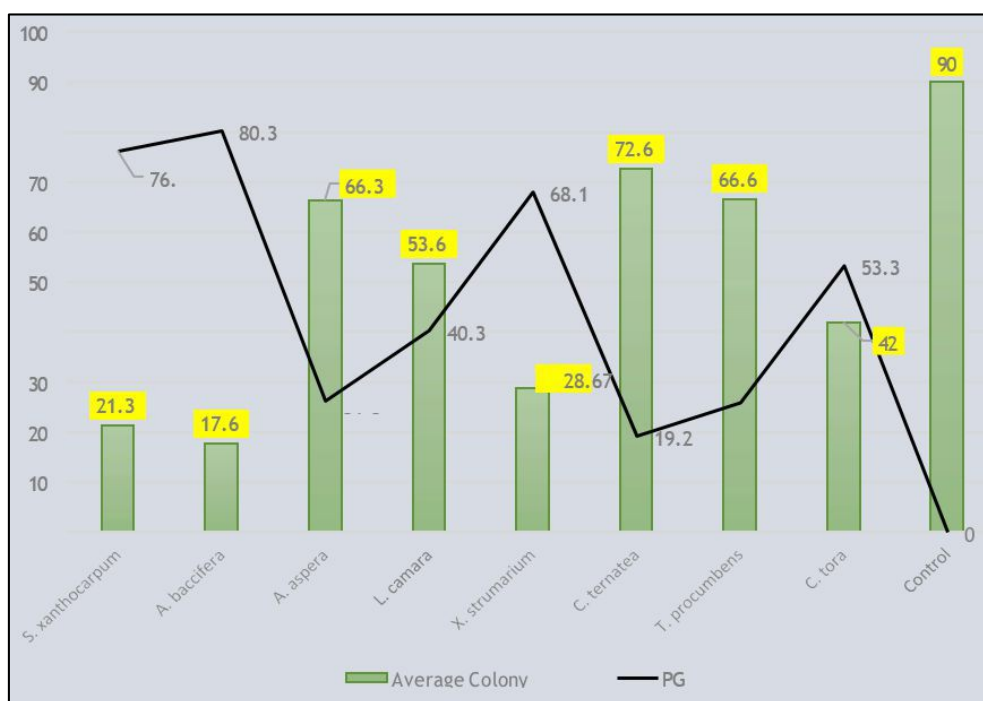


Fig 1: Average colony diameter and growth inhibition of FOC when evaluated with aqueous formulations of plants

To check the efficacy of the most effective phytoextract on seed germination and seed protection.

The efficacy of *A. baccifera* extract on the germination and protection of chickpea seeds (local) against *Fusarium* sp. at 0.5% concentration was determined using blotter method. The fungicide Thiophanate methyl (@ 3 g/kg seeds) was used as chemical check. The seeds were soaked in *A. baccifera* extract for 1 hour and the fungicide was mixed with the seeds in the required concentration. Prior to treatment with the fungicidal substances, the seeds were treated with the spore suspension of *Fusarium* sp. (1×10^4 spores/ml). The observations were taken after five days of incubation. The data regarding these are presented in Table 2 and depicted in Figure 2.

Both the treatments showed excellent germination of the seeds with well developed growth of radicle and plumule. The highest germination was observed in case of treatment with Thiophanate methyl (100 %), followed by treatment with *A. baccifera* extract (99.17 %). Both the treatments were statistically at par. Poor seed germination was observed in case of control (19.17 %).

Both the treatments showed considerable reduction in disease incidence with respect to the control. The lowest percent disease incidence was found in case of treatment with the fungicide Thiophanate methyl (20%), followed by treatment with *A. baccifera* extract (20.83%). Thus, the percent disease control in case of Thiophanate methyl was found to be 80% and in the case of treatment with *A.*

baccifera extract was 79.17%. Both the treatments were found to be statistically at par. The disease incidence was highest in case of control (100 %). All the seeds that were not treated with the fungicide or plant extract were diseased

and unhealthy. Some of them were covered with the fungal mycelium too. The radicle, if emerged was shriveled and shrunken in many seeds.

Table 2: Evaluation of the efficacy of Thiophanate Methyl and *A. baccifera* extract on germination and protection of chickpea seeds against FOC

Treatments	PSG	PDI	PDC
T ₁	90.00* (100)**	26.56 (20)	80.00
T ₂	87.85 (99.17)	27.08 (20.83)	79.17
T ₃	25.93 (19.17)	90.00 (100)	-
S. Em. $\pm$	1.30	0.63	
C. D. at 5%	3.90	1.91	
C.V. %	4.67	3.25	

*Figures outside parenthesis are arc sin transformed values

**Figures inside parenthesis are original values

where,

PSG = Percent Seed Germination PDI = Percent Disease Incidence PDC = Percent Disease Control

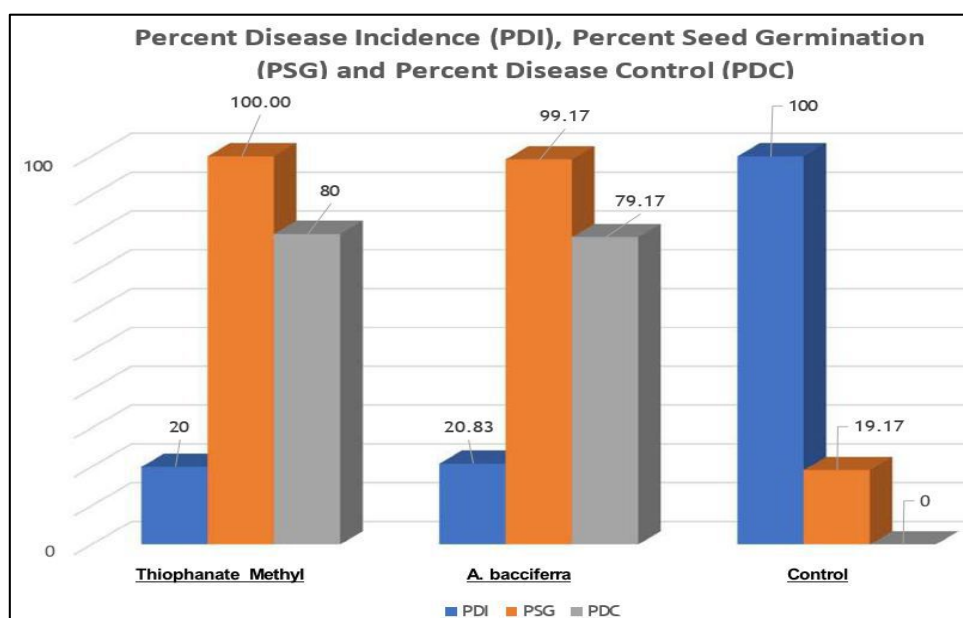


Fig 2: Evaluation of the efficacy of Thiophanate Methyl and *A. baccifera* extract on germination and protection of chickpea seeds against FOC

Thus, in this study, *Ammannia baccifera* L. proved to be a potential antifungal plant for the biological control of FOC. It is commonly found as a weed in rice fields. (Dash *et al.*, 2008). Known as Blistering *Ammannia*, this plant has been used in traditional medicine across India and China. Its secondary metabolites, primarily phenolic in nature, exhibit antimicrobial properties. Phenolic compounds are toxic to pathogens, as their hydroxyl groups disrupt cellular membranes and deactivate enzymes critical for survival. It has become well known for its antioxidant activities (Loganayaki *et al.*, 2012; Goodla *et al.*, 2019).

Conclusion

This study demonstrates the significant inhibitory effects of the crude extract of *Ammannia baccifera* L. on *Fusarium oxysporum* f. sp. *ciceris* (FOC), underscoring its potential as natural antifungal agent. Still, the limiting factor that remains are the screening techniques. More plants can be screened for their antimicrobial activities. There are no standardized extraction methods available for the

preparation of plant extracts. Also, the evaluation of antimicrobial activity is largely dependent on the inhibition zone assay and poisoned food technique and the other methods are not employed much. Most of the plants tested for antimicrobial activities have shown considerable results in case of *in vitro* experiments and future research should focus on field trials and formulation development to further assess the practical applications and efficacy of these plants in agricultural settings.

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