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Evaluation of the efficacy of some botanicals and bioagents against the wilt pathogen of spinach (*Spinacia oleracea*), *Fusarium oxysporum* f. sp. *spinaciae*

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Abstract
In vitro studies were carried out to determine whether certain botanicals and bioagents were effective against *Fusarium oxysporum* f. sp. *spinaciae*, the organism that causes *Fusarium* wilt disease in spinach (*Spinacia oleracea*) plants. *Argemone mexicana* and *Pithecellobium dulce* (Manila tamarind) were the botanicals, and *Trichoderma viride*, *Trichoderma harzianum*, and *T. hamatum* were the bioagents used in this study. All plant extracts and antagonistic organisms significantly inhibited the growth of *Fusarium oxysporum* f. sp. *spinaciae*. However, *T. harzianum* had the greatest inhibitory effect of the antagonists, followed by *T. viride* and *T. hamatum*, which have the least suppressive effect. *Pithecellobium dulce* and *Argemone mexicana* extracts had the strongest inhibitory effects.

Keywords: Spinach, wilt, botanicals, bioagents, *Fusarium oxysporum* f. sp. *spinaciae*

Introduction
Spinacia oleracea (Spinach) is an edible flowering plant in the family Amaranthaceae. It is a very common herbaceous plant in the tropical regions of the world. It was first cultivated by the Arabs and probably originated in southwest Asia. It is an annual dioecious plant and is best available during the winter. It grows rapidly, especially in spells of dry weather with bright sunshine, and has a tendency to bear seed quickly [1]. It is a common dietary vegetable that has various medicinal properties as it contains abundant levels of antioxidant compounds, is higher in iron, calcium, and vitamins than most cultivated greens, and is one of the best sources of vitamins A, B, and C [2].
Fusarium oxysporum f. sp. *spinaciae* (Sherb.) is the causal agent of *Fusarium* wilt of spinach (*Spinacia oleracea* L.), a serious disease of spinach worldwide. *Fusarium oxysporum* f. sp. *spinaciae*, is a fungus that can survive for many years in soil without a spinach crop. *Fusarium* wilt occurs in fields in patches and originates either at the early (seedling) crop stage or at the reproductive (adult plant) stage [3]. The fungus can survive on seed and cause disease in fields previously free of spinach wilt. Warm, acidic soils favor the pathogen. Wilt symptoms in the field include wilting of older leaves, stunting of plants, and shrinking and curling of leaves from the lower part of the plants that progressively move up to the stems of the infected plant. Plants finally become yellow and die [4].
Due to its soil-borne origin, ability to persist for longer periods of time without host plants, ability to form persistent dormant structures, and soil-borne nature, management of *Fusarium* infection is frequently a difficult task [5]. Utilizing resistant cultivars, crop rotation, synthetic fungicides, and fumigation have been the main methods used in recent decades to prevent and control soil-borne crop diseases [6]. Research areas in agricultural sciences now include the development of biological disease control and the hunt for novel, distinctive natural microbial phytopathogen antagonists [7]. However, there has been little success and little financial benefit from the use of soil fungicides. Alternative management approaches are therefore required for the environmentally friendly and long-term control of soil pathogens.

Material and Methods
Collection and authentication of test plant material
Plant materials of the selected plants for tasting *in vitro* efficacy against the isolated fungi from spinach were collected from JES College Campus Jalna, Maharashtra, India.

The plants with inhibitory properties were authenticated, and herbarium voucher specimens were prepared and deposited in the Herbarium, Department of Botany, JES College, Jalna, Maharashtra, India for future reference.

Preparation of Plant Extracts

In this study, aqueous plant extracts were used. Plant leaf extracts of *Argemone mexicana* and *Pithecellobium dulce* are used for the evaluation of antifungal activity against *Fusarium*. To obtain a sample, the powdered plant material from the leaf part was taken separately and subjected to the Soxhlet extraction procedure [8]. Preliminary phytochemical analysis was carried out for each solvent extract according to the standard procedure [9-10]. The extracted samples were kept in closed glass vials and refrigerated at 4 ºC when not in use [11]. Plant extracts were prepared and evaluated for their bioactivity using the agar dilution method [12].

Isolation and purification of the pathogen

The diseased plant showing the symptoms was washed thoroughly with tap water, and small pieces from infected parts 1–2 mm in dimension from the advancing margin of the spot, adjacent to healthy portions were cut with the help of a sterilized blade. These pieces were surface sterilized with 1% sodium hypochlorite solution for 30 seconds and finally washed well in three changes of sterilized distilled water to remove any trace of sodium hypochlorite. The pieces were then transferred aseptically to Petri plates containing Potato Dextrose Agar. Inoculated Petri plates were incubated at 26 ±1oC for three to five days and examined at frequent intervals to see the growth of the fungus. The pure colonies were picked and inoculated in a culture tube for further research work. Pathogen was identified on the basis of their morphological and cultural characteristics with the help of available literature [13].

Isolation, purification, and identification of antagonistic microorganisms

The isolation of bioagents was done by the serial dilution technique. 10 g of rhizospheric soil was dissolved in 100 ml of sterile distilled water to get a 10^{-1} dilution. 1 ml of soil suspension was taken from stock and added to 9 ml of sterile distilled water to get a 10^{-2} dilution. This is further repeated until a final dilution of 10^{-7} is obtained. 1 ml of each soil suspension was poured into sterilised Petri plates containing nutrient medium, incubated at 25 ± 1 °C, and observed at frequent intervals for the development of colonies of bio-agents. Bioagents were identified based on cultural and mycological characters described by Barnett and Hunter.

Dual culture technique

Using the dual culture methods of Morton and Stroube (1955) on PDA, antagonistic activities of the biocontrol agent were examined against the soil-borne plant pathogen *Fusarium oxysporum* f. sp. *spinaciae* (Sherb.). A 5 mm mycelial disc was obtained from the periphery of a 5-7 day-old culture of the pathogen on PDA, and it was positioned on a fresh PDA plate (3 cm from the bioagent centre). Next, fungal bioagents were placed 3 cm away from the inoculum of the pathogen. Three replication of each treatment were maintained with one control set without inoculating the bioinoculants. Then the plates were incubated at 26+1 °C. At the end of the incubation period, radial growth was measured. The inoculated plates with culture discs of pathogens without bioagents served as controls. After the 3rd, 5th and 7th days of incubation at 26+1 °C, radial growth of the pathogen and percent inhibition were recorded.

Determination of antifungal activity

The effectiveness of two plant extracts on the growth and inhibition of *Fusarium oxysporum* f. sp. *spinaciae* (Sherb.) was investigated. The inoculating needle was used to take *Fusarium oxysporum* f. sp. *spinaciae* into the extract-containing (poisoned) PDA in Petri dishes to check for its growth and PDA without extract served as the control. The extracts were then incorporated in 50 µl of different concentrations (5%, 10%, and 25%) into the agar. Each treatment was replicated 3 times. Each Petri dish was inoculated at 28±2 ºC for 7 days. The radial growth of the colony was recorded on the 3rd, 5th, and 7th days of intervals, and the percent inhibition of mycelial growth was calculated over control. The tests were carried out in triplicate. Per cent inhibition of mycelial growth over control was calculated by using the formula:

$$PI = \frac{C - T}{C} \text{ X } 100$$

Where, PI: is the percent inhibition over control, C: is mycelial radial growth in control plate, T: is mycelial radial growth in treatment [14].

Results and Discussion

Table 1: Preliminary Qualitative phytochemical screening of aqueous extracts of *Argemone mexicana* L

Sr. No.	Phytochemicals	Test	Result
1	Flavonoids	Shibita's reaction test	++
2	Phenols	Lead test	+++
3	Steroids	Salkowski's Test	+
4	Glycosides	Borntrager's	+
5	Saponins	Froth test	+
6	Terpenoids	Salkowski test	+++
7	Tannins	Ferric chloride test	++

Where (+) sign indicates presence of corresponding phytocomponents

Preliminary Qualitative phytochemical screening of aqueous extracts of *Argemone mexicana* (L.) results

The result of the present study is the outcomes of extracted contents of plant leaf extract (aqueous) tested for presence or absences of various phytochemicals (in qualitative form) are noted in Table 1. The results show that *Argemone mexicana* L. plant contains a maximum ten types of phytochemical groups, such as Carbohydrates, Alkaloids, Flavonoid, Phenols, Steroids, Glycosides, Saponins and Terpenoids. Phytochemical screening of *Argemone mexicana* L extracts reveals the presence of various secondary metabolites in them. Several researchers have evaluated the phytochemical properties of plant extracts by qualitative methods [15-16].

Table 2: Preliminary Qualitative phytochemical screening of aqueous extracts of *Pithecellobium dulce* L.

Sr. No.	Phytochemicals	Test	Result
1	Flavonoids	Shibita's reaction test	+++
2	Phenols	Lead test	++
3	Steroids	Salkowski's Test	--
4	Glycosides	Borntrager's	++
5	Saponins	Froth test	--
6	Terpenoids	Salkowski test	++
7	Tannins	Ferric chloride test	+

Where (+) sign indicates presence of corresponding phytocomponents.

Preliminary Qualitative phytochemical screening of aqueous extracts of *Pithecellobium dulce* L.

After the successful conventional hot-soxhlet extraction of the *Pithecellobium dulce* leaves, the preliminary phytochemical study revealed that the aqueous extract of *Pithecellobium dulce* L. contains alkaloids, flavonoids, phenols, and tannins (Table 2). Similar observations were reported for phytochemical screening of the plant, which revealed the presence of phytochemicals like tannins, flavonoids, and alkaloids by Kumar *et al*., 2013; Shanmugakumaraan *et al.*, 2008. The identification and purification of secondary metabolites found in active fractions are the next steps.

Table 3: Growth of *Fusarium oxysporum* f. sp. *spinaciae* on PDA incorporated with three different Concentrations of two plant extracts at 3rd, 5th and 7th of incubation

Treatments	Concentration (%)	Inhibition after 3rd Day		Inhibition after 5th day		Inhibition after 7th day	
		Radial Growth of pathogen (mm)	% Inhibition	Radial Growth of pathogen (mm)	% Inhibition	Radial Growth of pathogen (mm)	% Inhibition
Argemone mexicana	5	13	35	24	40	33	58.75
	10	17	15	21	47.5	31.5	60.62
	15	12	40	23.5	41.25	39	51.25
Pithecellobium dulce	5	11.4	43	19.4	51.5	29.7	62.87
	10	12.8	36	26	35	38	52.5
	15	9.4	53	17.8	55.5	27.3	65.87
Control		20	0	40	0	80	0

Effects of plant extracts on *Fusarium oxysporum* f. sp. *spinaciae*

Different concentrations of extracts of *Argemone mexicana* and *Pithecellobium dulce* were incorporated into agar at different volumes of 5, 10, and 15 percent, and the poisoned agar was inoculated with *Fusarium oxysporum.* After the third day of inoculation, the growth of the organism is reflected in the concentrations of 5, 10, and 15 of *Argemone mexicana* extract were 13, 17, and 12 mm. By the fifth day, the growth was 24, 21, and 23.5 mm, respectively. Similarly, on the seventh day, the growth was 33, 31.5, and 39 mm, respectively. The percent inhibition of *Fusarium oxysporum* due to the 5, 10, and 15% concentrations of *Argemone mexicana* extract incorporated into the growth media were 58.75, 60.62, and 51.25 percent, respectively, at 7 days of incubation. This observation shows that the growth of the target organism on the poisoned agar plate was reduced compared with the growth on the control plates, which were not poisoned with the extract of *Argemone mexicana* (Table 3). There was a significant difference in the growth of *Fusarium oxysporum* in different concentrations of *Argemone mexicana* extracts. The 10% was observed to be the most effective followed by 5% and the 15% was the least effective. Bhale *et al*., reported a significant reduction in the incidence of wilt disease in spinach by the plant extracts. At higher concentrations, mycelial growth was inhibited more than at lower concentrations. Gopal Reddy evaluated crude aqueous leaf extract of *Argemone mexicana* L for their antifungal efficacy on the growth of *Fusarium oxysporum* extracts in their finding revealed that higher concentrations of the extract showed greater inhibitory activity than the lower concentration.

After the third day of inoculation, the mycelium growth of the organism at different concentrations of 5, 10, and 15 percent of *Pithecellobium dulce* were 11.4 mm, 12.8 mm, and 9.4 mm. whereas on the fifth day, the growths of the target organism on the concentrations of *Pithecellobium dulce* were 19.4, 26, and 17.8 mm, respectively. Similarly, on the seventh day, the growth was 29.7, 38, and 27.3 mm, respectively. The percentage inhibition of *Fusarium oxysporum* due to the 5, 10, and 15 percent concentrations of *Pithecellobium dulce* extract incorporated into the growth media; was 62.87, 52.5, and 65.87%, respectively, at 7 days of incubation. This shows that the growth of the target organism was reduced when compared with the growth on the control plate, which was not poisoned with an extract of *Pithecellobium dulce.* Here the 15% was observed to be the most effective followed by 5% and the 10% was the least effective.

Table 4: *In vitro* bioefficacy of bioagents against *Fusarium oxysporum* f. sp. *spinaciae*

Tretment	Duration	Colony Dia. of test pathogen * (mm)	% Inhibition
T. viride	3rd Day	12.77	67.87
	5th Day	13.43	69.09
	7th Day	14.87	72.89
T. harzianum	3rd Day	19.87	83.09
	5th Day	20.23	84.87
	7th Day	23.78	86.24
T. hamatum	3rd Day	8.78	23.12
	5th Day	10.23	25.32
	7th Day	11.87	29.33
Control	7th Day	90	0

In vitro bioefficacy of bioagents against *Fusarium oxysporum* f. sp. *spinaciae*

Results showed that *Fusarium oxysporum* was refractory to all tested bioagents' fungistatic and antifungal effects, and that their use significantly reduced the growth of *Fusarium oxysporum* compared to the untreated control (Table 4). *Fusarium oxysporum* mycelium had a pinkish hue with fluffy white at the top. On the side of the media culture, antagonistic organisms like *T. viride*, *T. harzianum*, and *T. hamatum* were inoculated.

T. viride constrained the growth of the intended target organism, *F. oxysporum.* The colony diameter measured 12.77 mm on the third day. On the fifth and seventh days, it increased to 13.43 mm and 14.87 mm, respectively, which is significantly greater than the growth rates of the control plates. The mycelium of *T. viride* appeared greenish in color and fully occupied the plate, covering up the pathogen (Table 4).

The mycelium of *T. harzianum* was green with fluffy white patches, and it inhibited the growth of *F. oxysporum*. On the third day, *F. oxysporum's* mycelium growth was 19.87 mm, and it grew to a maximum of 23.78 mm on the seventh day. When compared to the control plate, there was a significant difference that revealed *F. oxysporum* growth was inhibited during these times (Table 4).

For *T. hamatum*, the mycelium appeared in a ring shape, greenish at the tip and white at the center. The growth of *F. oxysporum* on the third day was 8.78 mm, and on the seventh day, it was 11.87 mm; there were significant differences when compared with the control plate, but as compared to *T. viride*, *T. harzianum*, and *T. hamatum*, shows the least inhibition on *the* growth of the pathogen (Table 4).

Amongst the bioagents tested, *T. harzianum* was found most effective, showing the least mycelial growth (23.78 mm on the seventh day of incubation) and its highest inhibition (86.24%) of the test pathogen, followed by *T. viride* (72.89%) and *T. hamatum* (29.33%) in terms of percent mycelial inhibition. The results of the present study on the antagonistic effects of *Trichoderma* spp. against *F. oxysporum* are in accordance with those reported earlier by several workers such as Kurundkar reported on the effectiveness of *Trichoderma* species against *F. oxysporum* f. sp. *carthami*, the pathogen that causes the wilt of safflower, and discovered that isolates 29 and 33 had the least amount of pathogen growth when compared to others. Plant pathogens' mycelial growth was significantly inhibited by the species of *Trichoderma* [22].

Conclusions

In contrast to synthetic fungicides, our study showed that the use of aqueous plant extracts could be a useful, secure, and affordable method for controlling soil-borne diseases *(Fusarium oxysporum* f. sp. *spinaciae*). Additionally, there are benefits to using plant extracts as fungicides in agriculture because they naturally disintegrate and don't leave a harmful residue on plants. Our study also revealed the possibility of using regional isolates of *T. harzianum*, *T. viride*, and *T. hamatum* as biological control agents to shield bean plants from *Fusarium oxysporum* f. sp. *spinaciae*. Other practices that aim to enhance crop health and provide new standards of disease management where other techniques are ineffective could be incorporated into biocontrol programming.

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