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Bioactivity of methanolic plant extracts under *in vitro* conditions on inhibition of *Stemphylium vesicarium*, an incitant of Stemphylium blight in onion

S.U. Nabi*[1], G. Malik[1], R. Selvakumar[1], W.H. Raja[1], A. Sharma[1], D.B. Singh[1], M.A Sheikh[1], R Rasool[1] and M Shafi[2]

*[1]ICAR-Central Institute of Temperate Horticulture, Srinagar, 191132, J&K, India. [2]Division of Biochemistry, (SKUAST-J) Jammu, J&K, India. *E-mail*: sajad_patho@rediffmail.com*

Abstract

Onion (*Allium cepa* L.), an important vegetable and spice crop, is susceptible to Stemphylium blight incited by *Stemphylium vesicarium*. It causes significant losses (up to 80 %) in seed as well as bulb crops. The synthetic fungicides are the only option available to farmers for its management, which in long run may result in resistance development in pathogen. So there is a need to find novel strategies for management of this disease, hence the present study was devised to evaluate the antifungal efficacy of plant extracts from eight medicinally important plant species. The test fungus *S. vesicarium* was isolated from symptomatic leaf samples and was identified by characteristics of spore from available literature. Methanolic extracts of selected plants at three different concentrations (0.1, 0.5 and 1.0 %) were evaluated against *S. vesicarium* using poison food technique under *in vitro* conditions. The results showed that all plant extracts exhibited statistically significant antifungal efficacy from each other ($P < 0.05$). But *Origanum vulgare* at 0.5 and 1 % concentration exhibited highest antifungal efficacy (68.23 % and 81.3 % respectively). The importance of the present study lies in that the oregano extracts had the potential to manage the disease under field conditions after isolation of bioactive molecule and development of proper formulation. To the best of our knowledge this is the first kind of study conducted, where oregano has been reported to be effective against plant pathogen.

Key words: Onion, Stemphylium blight, *Stemphylium vesicarium*, plant extract, management

Introduction

Onion (*Allium cepa* L.) is one of the vegetable crops of global importance cultivated worldwide. Being a rich source of minerals, vitamins, dietary fibres, and antimicrobial anticancer elements, it is also known as protective food (Griffiths, 2002). It stands second in terms of annual world production among the 15 vegetables listed by FAO (Mohsin *et al.*, 2016). Its centre of origin is believed to be northern mountainous regions of Iran and Pakistan (Purseglove, 1972; Islam, 2006). Besides being consumed as vegetable, it is also used as spice and salad in most of the Asian countries (Chakraborty *et al.*, 2015). The main chemical constituent present in onion is allyl-propyl-disulphide, which is responsible for pungency and also acts as anti-carcinogen (Krest and Keusgen, 1999; Steinmetz and Potter 1996). India was second-largest producer and 4th largest exporter in 2018 (in dollar value) (Anon.). Nevertheless productivity of onion in India is very low, and one of the reasons is susceptibility of Indian genotypes to several biotic and abiotic stresses under different agro-climates of the country, which affect not only its production but also processing and marketing. It is prone to 66 diseases caused by bacteria (10), fungi (7), viruses (3), mycoplasma (1), parasitic plant (1), and by other pathogens (Schwartz, 2010). Among various diseases under temperate conditions, Stemphylium blight caused by *Stemphylium vesicarium* is most important disease, which causes severe damage and significant losses up to 80 to 85 % (Tomaz and Lima, 1988). Although the disease is managed by application of several new generation fungicides (Cho *et al.* 1995), most of them are systemic in their mode of action, which can lead to development of resistance in pathogen and disturbance in complete microbiome of whole ecosystem (Sajad *et al.*, 2017). In the present scenario, due to the increased demand for organic products, there has been a change from the use of conventional systems to organic systems using alternative approaches for disease management (Hwang *et al.* 2009; Kwak *et al.*, 2012). Botanicals are substantial sources to replace synthetic fungicides as these are environmentally safe and have recently gained importance due to their efficacy against phytopathogens and cost-effectiveness (Kokab *et al.*, 2018). Nature has bestowed us with numerous medicinally important plants, and more than 250,000 higher plant species have potential antimicrobial properties due to the presence of inhibitory bioactive compounds. During last several decades, researchers have evaluated plant extracts against plant pathogens, valuable results have been achieved, and some commercial botanical formulations have been prepared and marketed (Zaker, 2016). Several studies have been conducted using botanicals for management of diseases like Phytophthora blight of pepper, spot blotch of wheat, leaf blotch of barley, purple blotch of onion (Yoon *et al.*, 2010, Bhadhur *et al.*, 2016). Taking into consideration the importance of botanicals, the present study was conducted to screen out selected botanicals to determine the antifungal efficacy against *S. vesicarium* and management of Stemphylium blight of onion.

Materials and methods

Isolation, purification, and identification of the pathogen: Suspected diseased leaf samples were collected from onion field of ICAR-CITH Srinagar, during 2017-18 growing season. Small symptomatic along with healthy portions of onion leaves were surface disinfected in 2 % sodium hypochlorite solution, rinsed twice in sterilized distilled water and placed on potato dextrose agar (PDA) plates and were incubated for 7 days at 25 ± 2 °C (Galvez, 2016). The culture was purified by single spore isolation (Johnston & Booth, 1983) and was identified by comparing with available literature of *Stemphylium* spp. described by Simmons (1969).

Collection of plant material (Botanicals): Eight plant species were selected after considering their potential to control plant pathogens based on a literature review (Mishra and Gupta 2012, Wani *et al.*, 2017). Plant species were collected from the medicinal plant field gene bank maintained at ICAR-CITH, Srinagar. The collected plant species with their botanical/local name along with part used for evaluation are listed in Table 1.

Table 1. Plant species along with their part used for evaluation

English/Local Name	Botanical Name	Parts Used
Artichoke	*Cynara scolymus*	Leaves
Artemisia (Tethwen)	*Artemesia bisinthium*	Leaves
Geranium	*Geranium maculatum*	Leaves
Rosemary	*Rosemarinus officinalis*	Leaves
Walnut (Doon)	*Juglans regia*	Hull
Oregano	*Origanum vulgare*	Leaves
Iris	*Iris nepalensis*	Leaves
Rhubarb (Pambchalan)	*Rheum emodi*	Rhizome

Preparation of plant extracts: The plants parts (leaves, rhizome, hull) of 8 plant species (Table 1) were shade dried for one week, ground to powder form using grinder mixer, and sieved through 1 mm mesh, after which these were subjected to extraction. Thirty g of the ground and sieved material of each plant species were placed separately in 300 mL methanol (polar solvent) for 24 hours for the methanol extract. The extract was filtered through filter paper, and supernatant was taken as standard plant extract solution (100 %). The supernatant was concentrated by complete evaporation of solvent using a rotary evaporator (Mishra and Gupta 2012, Wani *et al.*, 2017). The final weight of the pellet was measured and dissolved again in 5 mL of solvent for storage and future use.

***In vitro* evaluation of extracts on mycelial growth of pathogen:** The plant extracts were evaluated (*in vitro*) using poison food technique (PFT) at three different concentrations, C_1-0.1 %, C_2-0.5 % and C_3-1.0 % against *S. vesicarium*. Extracts were subjected to a temperature of 50 °C in water bath to avoid contamination and were incorporated into 100 mL of melted potato dextrose agar (PDA) media. The media was gently shaken for thorough mixing of the extract so that the final concentration of the media was 0.1 %, 0.5 %, 1.0 %, and 20 mL media were poured into Petri dishes of size 9 cm (Kumar *et al.*, 2012). The PDA plates containing the plant extracts were inoculated aseptically by transferring 5 mm diameter agar disc of 7 days old culture of the *S. vesicarium* to the centre. Three replications were maintained for each treatment, and the respective solvent without any plant extract served as control. All the inoculated Petri plates were incubated at 25 ± 2 °C temperature. The radial growth of the test fungus in the treated plates was measured in all treatments after 7 days of inoculation. The percent growth inhibition of fungal growth was estimated by using the formula as below given by Vincent (1927).

$$\text{Growth inhibition (\%)} = \frac{C-T}{C} \times 100$$

Where,

C =Colony diameter in control. T=Colony diameter in treatment

Statistical analysis: The experiment was conducted in complete randomised design (CRD) with three replications, and data were expressed as a percentage of the control to standardize comparisons between treatments. A significant difference was determined using one way analysis of variance (ANOVA) using Statistical Analysis Systems (SAS 2000) software. In case of significant difference, means were separated using Duncan's Multiple Range Test at $P \leq 0.05$.

Results and discussion

Identification of pathogen based on morphological characteristics: Stemphylium leaf blight is one of the most serious diseases of onion and garlic and due to the presence of high level of genetic variation in its causal organism; it is reported to be different species depending on the region (Basallote *et al.*, 2004; Zheng *et al.*, 2008). During our study, the symptoms observed on leaves and flower stalks were small, yellowish flecks with purple colored margins. The cultural characters of fungus such as colony colour, colony texture, and radial growth recorded on the seventh day of inoculation revealed greenish-brown to dirty white colony colour, circular margins, and 25-30 mm in diameter after 7 days of inoculation. Morphological characters like mycelium, shape, colour, length, width and septation of conidia and conidiophore studied using microscopy revealed that the mycelium was septate, conidia having brown colour with oblong to ovoid shape and constricted at middle septum, with horizontal and vertical septa ranged from 3.6-5.0 and 1.4-3.2, respectively, the length and width of conidia varied from 59.3-71.2 µm and 9.3-12.5 µm, respectively (Fig. 1). The characters were similar to those described by Simmons (1969) for *S. vesicarium,* hence confirmed the blight symptoms observed on onion in the field were associated with the pathogen. The taxonomy of this genus has always been controversial, and the classification has been revised based on molecular and morphological methods (Inderbitzin *et al.*, 2009; Simmons, 1969).

Inhibition of *S. vesicarium* by selected plant extracts:

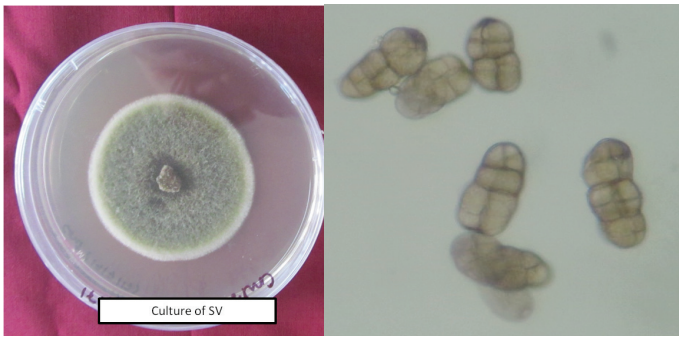

Fig. 1. Seven days old culture and conidia of *Stemphylium vesicarium* (SV) observed under bright field compound microscope (400 X)

Management of fungal pathogens using plant extracts is cost-effective and eco-friendly approach. Various plant extracts were evaluated during our study. The results presented in Table 2 revealed that the mycelium inhibition of the *S. vesicarium* by various extracts ranged from 12 to 81 %. Statistically significant differences were observed between the botanicals and among the concentrations used against *in vitro* inhibition of *S. vesicarium*. The oregano extract at 1 %, 0.5 % and 0.1 % concentrations gave 81.3 %, 68.23 %, 47.71 % inhibition, respectively, over control (Fig. 2a, b). In similar studies, Mishra and Gupta (2012) and Kumar *et al.* (2012) reported that the botanicals could inhibit the mycelial growth of *S. vesicarium* up to 57.31 % and 66.5 %, respectively. Similar results on antifungal activity of aqueous extracts of different botanicals have been reported against onion by Prasad and Barnwal (2004), and also reported on other hosts against the same pathogen by Subedi *et al.*, (2015). With the advent of chemical pesticides it was thought that a permanent and reliable solution of plant diseases would be achieved, but it was realized soon that the intensive and indiscriminate use of synthetic pesticide in agriculture is not safe to the environment as the toxicants cause soil, water pollution, animals and food contamination; poisoning of farmers; elimination of non-target organisms and have harmful effects on human beings (Stangarlin, 2011). Taking into consideration the ill effects caused by synthetic pesticides, there is increasing need for alternative pesticides, which are biodegradable, eco-friendly, toxicologically safe, have greater selectivity, efficacious, and suitable for use in integrated pest management programmes (Samarrai, 2012). Since these are easily and rapidly degradable in sunlight, air, and moisture and by detoxification enzymes, have less persistence and reduced risk to non-target and beneficial organisms. However precise timing and/or more frequent applications may be necessary (Rice, 1983). Mountains of Kashmir valley are natural sources for medicinal plant wealth and researchers need to evaluate as much as against plant pathogens for safe management of plant diseases. In our study, preliminary screening of some plant species showed a strong potential for antifungal efficacy. It is evident that the oregano plant extract has strong potential to act as botanical fungicides for management of stemphylium blight after proper evaluation in field related to its toxicity to pathogen and safety of plants.

Plants are a great source of thousands of new, useful, and biologically active molecules of great diversity, which have inhibitory effects on various types of microorganisms. Our study confirmed the extracts from eight plant species showed

Table 2. *In vitro* inhibition of *S. vesicarium* using various methanolic plant extract (after 7 days of inoculation)

Plant species	Part used	Extract Concentration		
		C_1	C_2	C_3
Artichoke	Leaves	12.77^{d}	21.09^{d}	22.03^{de}
Artemisia (Tethwen)	Leaves	16.10^{dc}	16.18^{e}	23.16^{d}
Rosemary	Leaves	14.38^{d}	21.06^{d}	34.5^{c}
Oregano	Leaves	47.71^{a}	68.23^{a}	81.3^{a}
Iris	Leaves	27.44^{b}	27.05^{c}	22.54^{de}
Geranium	Leaves	21.01^{c}	37.87^{b}	44.3^{b}
Rheum (Pambchalan)	Rhizome	15.20^{dc}	14.83^{e}	19.06^{e}
Walnut	Hull	13.33^{d}	14.23^{e}	14.03^{f}

Means, within columns, followed by the same letters in superscript are not significantly different at $P < 0.05$ (Duncan's Multiple Range test).

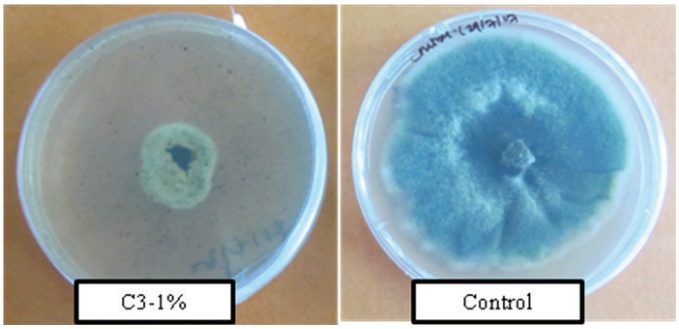

Fig. 2a. Inhibition observed over control at 1 % concentration under *in vitro* conditions using oregano plant extract

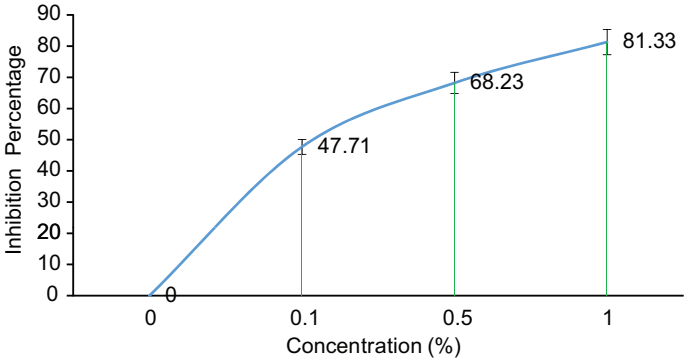

Fig. 2b. Effect of oregano plant extracts at different concentrations over control (0) on *in vitro* inhibition of *S. vesicarium*

inhibition up to various levels, and among them the oregano plant extract at different concentrations inhibited the *S. vesicarium* up to significant extent. Hence these extracts have potential to manage the disease in field as well. The future prospects include the isolation, characterisation and identification of the molecules present in the oregano, which are responsible for inhibition of *S. vesicarium*. Also in future research efforts need to be directed not only towards the development and application of known botanicals but also on screening large number of plants, isolate new and novel bioactive molecules which have potential to control spectrum of diseases.

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