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Screening of Alfalfa Germplasm and Evaluation of Fungicides Against *Sclerotinia sclerotiorum* Causing Stem and Crown Rot

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Alfalfa (*Medicago sativa)*, a member of the Fabaceae family, is regarded as the queen of fodder. It is also the oldest and most significant forage crop in the world. Alfalfa is susceptible to various bacterial, fungal, and viral diseases, with stem and crown rot, caused by *Sclerotinia sclerotiorum*, posing a major threat. These diseases significantly reduce alfalfa productivity. Significant annual yield losses are anticipated due to this disease. Since sclerotinia disease affects a variety of hosts, it is becoming a significant global issue, with annual reports of significant yield losses. Given these significant losses, this research primarily focused on disease management through screening for resistant alfalfa cultivars. In the greenhouse, pathogenicity was confirmed by following the Koch's postulates. Five alfalfa germplasm types were evaluated for resistance sources. The best method for managing stem and crown rot disease was V3. Disease severity data was documented using the Welty disease rating system (grades 0-6). To compare the means of several alfalfa varieties, the Least Significant Difference Test (LSD) was applied at a 5% probability level. Statistical analysis was performed using a Factorial design within a Randomized Complete Block Design (RCBD).

Keywords: Alfalfa, *Medicago sativa*, fabaceae family, *Sclerotinia sclerotiorum*, Koch's postulates, Least Significant Difference Test (LSD), Randomized Complete Block Design (RCBD).

INTRODUCTION

Alfalfa was introduced to New Mexico, California, Arizona and Texas by catholic missionaries "By 1836, a number of regions in the southwest of the USA were cultivating alfalfa. Nevertheless, during the days of gold rush "Chilean clover" was introduced in California that gained a considerable significance (Bolton et al., 1975). On the other hand, it is also known that alfalfa cultivation is known to be a more profitable business than gold panning. Alfalfa has made its significant entry into California from Chile, a 100 years ago, then the crop has made its significance in Georgia in 1736, North Carolina in 1739 and in New York in 1791 (Bolton et al., 1975). Due to high acidic soils and higher humidity level in the atmosphere of Eastern states, lucerne has faced difficulties in survival. Taxonomically, alfalfa belongs to Plantae kingdom and sub-kingdom is *Tracheobionta*, and its class is *Magnoliopsida* and its Sub-class is *Rosidae* and belonging to order *Fabales* and the family is *Fabaceae* (pea family) and *Magnoliopsida* is its class and the genus is *Medicago* and specie name is *sativa*. Alfalfa is a bushy, deeply tap-rooted perennial and short-term crop. Alfalfa stems bear both simple, unifoliate first leaves and alternately arranged, pinnately trifoliate leaves. It has a simple and unifoliate first foliar leaf, and alternately, pinnately trifoliate leaves are arranged on stems. The stem of alfalfa grew up to an altitude of 1m from a crown and is erect. The shoots of alfalfa form reproductive and vegetative organs and are unstipulated. Alfalfa has a variety of yellow, variegated, orange, violet, gray, or purple floral colors. Raceme of alfalfa is axillary and consisting of a number of florets (Barnes, 1972). It was introduced many times in the 1700s and 1800s into North America, and now it is grown across the continent (Russelle, 2001). Alfalfa is known to be the fourth most commonly cultivated crop in the United States, covering an area of more than 9.3 million in 2003 (USDA-NASS, 2004). This is a persistent crop usually cultivated for four years (one year of establishment plus three years thereafter). Depending on the location, Alfalfa can be harvested up to four times by its stem cuttings near to the surface level. The annual yield of alfalfa across the United

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States is 7.8Mg dry matter and may vary from 3.4Mg per hectare in North Dakota to 18.5Mg per hectare in Arizona. And in 2003, alfalfa is harvested more than 68 million metric tons annually (USDA-NASS, 2004). It is concluded that genetic yield potential can increase the yield of the most of the crops up to 20-23 percent by comparing actual yields (Boyer, 1982). The fluctuations in the yield is due to the disease causing by weeds, pathogens and pests (known as biotic stresses) and also by abiotic stresses. The annual loss of worldwide food production can be estimated by acknowledging 32-42 percent lost due to weeds, pathogens and insects (biotic agents) and it is approximately US$ 500 billion. The loss due to insects, bacterial rots and fungal attacks during storage is 7-20 percent which is US$120 billion out of the total worldwide annual food production is US$ 1.3 trillion. The crop losses due to the attack of the pathogens in the developing countries is 23 percent in cereals and 7 percent in the developed countries (Oerke et al., 1994). Globally, it is considered that weeds are also taking or playing a major role in crop failure and creating a devasting situation worldwide. About US$120 billion losses are expected to cause by drought, shortage in nutrients, flooding, frost and in anoxia and air toxicity (abiotic causes) which is approximately 7-20 percent. Therefore, by analyzing the present situation, there is a need of the developing disease resistant varieties, which are resistant to biotic as well as abiotic stresses. In July 1982, Stem and Crown Rot Disease of Alfalfa caused by a species named *Selerotinia* were observed in several broadcast alfalfa stands used for seed production in the Touchet-Gardinia region near Walla Walla, southeast Washington. Alfalfa corona and stem rot, caused by S. sclerotiorum is known to occur in cool, humid European and North American regions (Adams & Ayers, 1979; Graham, 1979; Graham et al., 1972). Yet, with its dry and hot desert climate, alfalfa fields in eastern Washington were not supposed to occur. Nevertheless, in April, May, and June of 1982, the dense plant canopy combined with the cool and wet conditions provided near-optimum conditions for a significant occurrence of this disease activity. Several fields in this development area displayed substantial browning and dieback caused by *Sclerotinia* during June and July. There are no *Sclerotinia* resistant alfalfa cultivars, and the only methods of control are cultural practices. *Sclerotinia sclerotiorum* is known to be a serious threat towards a number of crops including alfalfa and many other forage legumes growing in the countries of temperate climates. This is known to be a serious because of causing devasting losses such as thinning of alfalfa stands and also by lowering alfalfa yields by causing Stem and Crown Disease of Alfalfa (Elgin et al., 1988). This disease can damage alfalfa plants of all ages but it can cause severe attack during the s of seedling in the temperate cool climate with humid conditions (Kanbe et al., 1997). This harm normally becomes evident in the spring after vegetation is resumed. If cultivars are particularly vulnerable, dead plants become evident only after vegetation is resumed. Later in the spring, the plants can tolerate the pathogen by slowing the symptoms of disease development like damage crowns as well as low number of wilting plants (Pratt & Rowe, 1995). Lucerne varieties vary greatly in tolerance to Stem and Crown Rot disease as compared to other crop plants (Khan, 2002; Pratt & Rowe, 1998; Pratt, 1996). So, *S. sclerotium* may be visible in fields before symptoms of Sclerotinia stem rot occur on alfalfa plants. As the disease Stem and Crown Rot progress visible symptoms of water-soaked lesions are shown on the infected stems of the plants that rapidly grow at the top to bottom of the stem and also on the infected nodes. Infected stems changed their color from tanned to bleached, and on roots, seeds, petioles and sometimes leaves, too, lesions can occur. As the disease progress the heath of the plants worsen that can lead it to towards lodging wilting and ultimately to the death of the plant. The attack of the *S.sclerotiorum* on the field crops can easily be detected by examining the symptoms like white, cottony, mycelial and mold like growth appearing on the infected stems and leaves. Due to the attack of the fungus sclerotia are grown inside or outside of the pods and stems of the plants. Generally, it can be said that the signs and symptoms of the disease progress caused by S. sclerotiorum can be detected and distinguished from other diseases of the crops (Grau & Hartman, 1999; Grau et al., 2004). Complete Stem and Crown rot control is a difficult challenge and impossible to achieve in all crops because a number of challenges are needed to manage the disease progression. To increase the yield and to control the disease progression, the use of chemicals and fungicides are applied (Woodward et al., 2015). Calcium salts have also been shown to minimize in vitro mycelial growth and to reduce the incidence and severity of various pathogenic infections (Chardonnet et al., 1999). Similarly, host resistance to the disease can also dramatically increase crop production in fields with extreme disease history. After data concerning accurate disease detection, efficacy of possible fungicides against S. There was a shortage of sclerotiorum and pathogen-resistance sources in alfalfa cultivars. The goals of this analysis were thus to describe the *S. sclerotiorum* causing stem and crown rot in alfalfa, and establishing a disease issue management strategy. The main aims of this research were to Evaluated germ plasm of alfalfa against *Seclerotinia sclerotium*.

MATERIALS AND METHODS

***Pathogenicity Test*:** Pathogenicity test was performed by following the Koch's postulates for the conformity of the pathogen present on the plants such as Pathogen must be present on the host plant, Isolation of pathogen from the host plant, Inoculation of pathogen to the healthy host plant for the

for the symptoms development and compared with diseased plant and Re-isolation of pathogen from inoculated plant.
Pathogenicity test of the isolate has been checked. In the net house, the alfalfa seeds were sterilized at the surface and sown in circular earthen pots filled with sterile soil. When the seedlings were 4-week-old, the fungal colonies formed on the PDA plates were inoculated into the stem bark by applying an artificial cut of about 10cm above the ground level with a blade and sealed with moistened cheese cloth. Twenty plants were inoculated with isolates, and inspections included 20 plants Subsequently, the inoculated plants were covered with polythene bags and placed in a dark growth chamber for 2 days at ±23-25 ° C. Then the inoculated seedlings were moved to a net house and grown for 20 days before observation. The isolate's pathogenicity was also tested on leaves by inserting a fungal mycelial plug on the leaf surface in a similar manner. The re-isolation of the fungus into the healthy plants is done by to confirm and compare the symptoms of the original pathogen.

Figure 1. Sowing of crop for Pathogenicity Test.

***Maintenance of Plant Material*:** Under good growth conditions, plant material was preserved. Plants were still carefully watered during the entire duration of experiments. And the plants were placed at ±25 to 35°C after inoculation to avoid the risks of water logging and drying out at ambient temperatures and also avoid over irrigation and under irrigation.
***Screening of Alfalfa germplasm for the source of resistance against S. sclerotiorum*:** For screening of alfalfa against Stem and Crown rot disease, seedlings of different varieties/ lines were obtained from the market and were grown in research area of Department of Plant Pathology, University of Agriculture Faisalabad.
***Collection of the Germ Plasm*:** A disease-free nursery of alfalfa seedlings, were taken from market, and grown in sterilized soil of experimental field in the Department of Plant Pathology University of Agriculture Faisalabad using RCBD (Randomized Complete Block design) and the seeds are placed with the spacing of 7 to 9 inches in rows with the drill.
***Artificial inoculation*:** The plants were regularly watered and maintained for inoculation with replications. The following method of inoculation is applied.

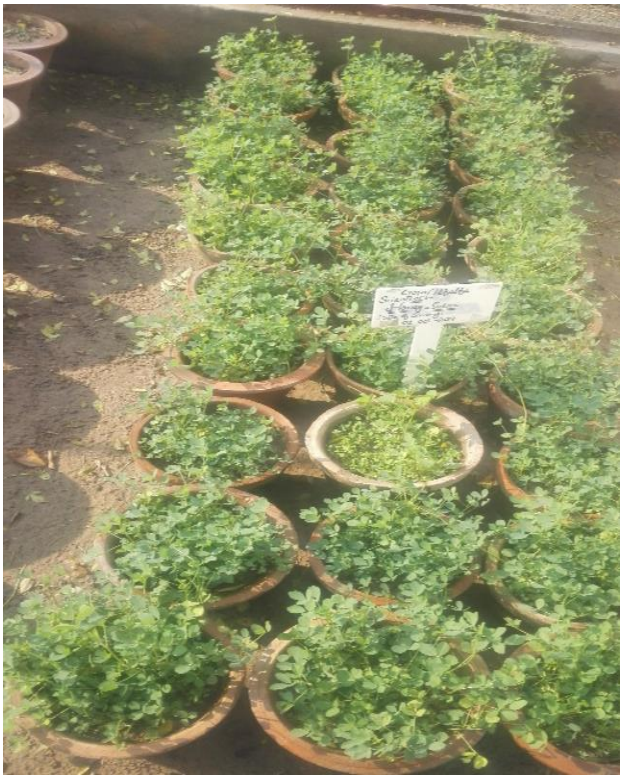

Figure 2. Maintenance of Plant Material during the entire duration of experiments.

***Inoculation with sclerotia*:** Sclerotia was harvested from 15 d old culture, washed with distilled water and placed at soil level near the stem base by causing injury with and without injury using caborundum powder. These sclerotia were coated with sterile wet cotton swab, and for one-week inoculated plants were coated with bags of polythene. Plants in pots were regularly examined for disease growth, and data were collected 15 d following inoculation. Effects of burial depth on sclerotic germination and development of apothecals. There have been attempts to germinate pathogen sclerotia in sand, clay soil and a combination of sand and clay soil (3:1). All substrate preparation and sterilization of 1.1 kg / cm2 at 121.6 C for 60 minutes. The sterilized substrates were filled and repeated four times in earthen pots (30 cm diameter). Fourty sclerotia have been sown at varying depths of 0, 2, 4, 6 and 8 cm in each pot. The pots were regularly watered at an interval of 2 d per month to provide the necessary humidity. They kept the pots at 20-24 C. Observations on percentage

germination and development of apothecia of sclerotia were reported at weekly intervals up to 90 days.

***Evaluation of resistance to SCSR*:** The following table is used to evaluate the alfalfa germplasm against stem and crown rot disease.

≤3.0 = resistant (R); >3.0 – 4.0 = medium resistant - resistant (MRR); >4.0 – 5.0 = medium resistant (MR); >5.0 – 6.0 = medium susceptible (MS); >6.0 – 7.0 = medium susceptible - susceptible (MS-S); >7.0 = susceptible (S) (Welty et al., 1978).

Table 1. Disease Scoring Scale.

Grade	Amount of disease (PDI)	Reaction
0	No Infection	Immune
1	1.0-3.0	Resistant
2	3.1-4.0	Medium resistant -resistant
3	4.1-5.0	Moderate resistant
4	5.1-6.0	Moderate susceptible
5	6.1-7.0	Medium susceptible-susceptible
6	>-7.1	Highly susceptible

***Meteorological conditions*:** Two weeks after planting, resistance to SCSR was assessed. Resistance of alfalfa accessions to varying oxalic acid concentrations. To restart alfalfa. The seriousness of the disease was calculated on a scale of 0-9 ratings, where 1 is the lowest.

***Statistical Design*:** The recorded data was subjected to analysis of variance (ANOVA) and LSD test at 5% level of significance (Jayachandran, 1983).

***Experimental Design and Statistical analyses*:** A number of experiments were carried out in field conditions, so it is reasonable to use Randomized Complete Block Design (RCBD). Results at the 5 percent significance stage were statistically analyzed.

RESULTS

***Completion of Koch's Postulates for Pathogenicity Confirmation*:** On the predominant isolation of *S. sclerotium* isolate (GenBank accession number FMB 0123) from the culture obtained from Fungal Molecular Biology Laboratory Culture Collection University of Agriculture Faisalabad (FMB-CC-UAF) was inoculated with the sclerotia of the pathogen in the surface of soil line of alfalfa grown in pots in Green House. After the appearance of the symptoms on the alfalfa plants, the symptoms were carefully examined and compared with the symptoms that appeared on the Stem and Crown rot of alfalfa caused due to *S. sclerotium*, as appeared in the figure 3.

The diseased samples were then taken from the alfalfa plants and then isolated on the petri plates for pathogen confirmation and in such a way pathogenicity test was accomplished. In method of inoculation, plant infection occurred by sclerotia. Typical signs of stem rot disease turned up on the plants in both cases. When the inoculum was positioned directly on the stem of the plant, a higher percentage of infection was reported. Mycelial disc inoculation was found to be serious, causing 70% infection in injured plants and 40 percent infection in un-injured plants.

Figure 3. Symptoms of the Stem and Crown rot of alfalfa caused due to *S. sclerotium*.

Although it was found that the sclerotia used as inoculum was less infectious. Symptoms of disease emerged as thin, greyish, water-soaked lesions 4 to 6 day after inoculation, which gradually grew to stem length and formed into patches of soft rotting tissue. When the diseased stem was broken, stem covered with whitish mycelial mats and black color of sclerotia in pith of varying sizes was observed on these patches. Defoliation of the leaves and death of the plant branches were observed as the disease progressed. Pathogen developed similar symptoms in both uninjured and injured plants as found in naturally infected crops but infection was higher for injured plants. Stem is wrapped in whitish mycelium. The infection of plants occurred by method of inoculation using sclerotia, typical symptoms of stem rot disease appeared on the plants. A higher percentage of infection was recorded when the inoculum was directly placed on the stem of the plant. Symptoms of disease appeared 4 to 6 days after inoculation as small, grayish, water-soaked lesions, which rapidly enlarged to stem length and developed into patches of soft rotting tissues. Stem covered with whitish mycelial mats and black color of sclerotia in pith of varying sizes were observed on these patches when the diseased stem was split. Defoliation of leaves and death of branches of plants were noticed with advancement of the

disease. Pathogen produced similar symptoms as observed in naturally infected crop.

This reality indicates the injury is predisposing plants to fungal attacks. Re-isolation from lesions formed on plants that were artificially inoculated resulted in the same fungus that was previously isolated from plants that were naturally infected. In this way the pathogenicity of the pathogen was confirmed by isolation, inoculation and re-isolation of the same fungus.

***Sclerotia germination and apothecial production*:** The pathogen *S. sclerotium* infects the alfalfa plants by ascosporic inoculum or by mycelial infection. The result showed that apothecary development (carpogenic germination) was higher when sclerotia was inoculated at the soil surface but with the increased burial depth in each substratum the rate of infection also decreases. All the substrates, however, have developed apothecia. A substantial decrease in the percentage of sclerotia generating apothecia with rising burial depths was also shown by the results of our analysis. This suggested that deep ploughing of infested fields could be a useful activity to reduce the pathogen's inoculum.

***Evaluation of alfalfa germplasm against stem and crown rot disease for the source of resistance*:** The data recorded on the basis of the visible signs or appearance of symptoms on alfalfa varieties in field due to Stem and Crown Rot Disease for the evaluation of resistant and susceptible cultivars against stem and crown rot disease.

Table 2. The screening results of alfalfa varieties against stem and crown rot.

Grade	Amount of disease (PDI)	Reaction	No. of varieties
0	No Infection	Immune	Nil
1	2.0-3.0	Resistant	Nil
2	3.1-4.0	Medium resistant - resistant	1
3	4.1-5.0	Moderate resistant	1
4	5.1-6.0	Moderate susceptible	2
5	6.1-7.0	Medium susceptible-susceptible	1
6	>-7.1	Highly susceptible	Nil

Table 3. Analysis of variance table for disease.

Source	DF	MS	F	P
Replication	2	0.1935		
Variety (V)	4	28.8159	4363.01	0.0000
Week	6	0.9182	139.02	0.0000
V * Week	24	0.0073	1.11	0.0355
Error	68	0.0066		
Total	104			

Grand Mean = 5.2249; CV = 1.56

Almost each leave and stem of alfalfa germplasm had rotted after two weeks of inoculation of *S. sclerotiorum*. Growth of the plants with the germination of pathogen with three alternative replications were observed on daily basis. In the first observation, cultivars did not show any evidence of symptoms**.** The screening results of alfalfa varieties against stem and crown rot concluded that no variety of alfalfa out of 5 was completely resistant against *S.sclerotium*, while medium resistant to resistance was showed by **V3** which is on Grade 2. Respectively, the variety named **V2** was found moderate resistant and placed on Grade 3, **V2** and **V5** varieties were found moderate susceptible and placed on Grade 4, and the remaining variety such as **V4** was medium susceptible to susceptible and placed on Grade 5.

Table 4. LSD All-Pairwise comparisons test of disease for variety.

Variety	Amount of disease (PDI)	Grade	Reaction
V1	4.5881 D	3	Moderate Resistant
V2	5.6200 C	4	Moderate Susceptible
V3	3.5357 E	2	Medium Resistant-Resistant
V4	6.5143 A	5	Medium Susceptible-Susceptible
V5	5.8662 B	4	Moderate Susceptible

Alpha = 0.05; Standard error for comparison = 0.0251
Critical t value = 1.995; Critical value for comparison = 0.0500
Error term used: Replication*Variety*Week, 68 DF
All 5 means are significantly different from one another.

Table 5. LSD All-Pairwise comparisons test of disease for week.

Weeks	Mean
1	4.8900 G
2	4.9800 F
3	5.1053 E
4	5.2333 D
5	5.3500 C
6	5.4567 B
7	5.5587 A

Alpha = 0.05; Standard error for comparison = 0.0297
Critical t value = 1.995; Critical value for comparison 0.0592
Error term used: Replication*Variety*Week, 68 DF
All 7 means are significantly different from one another.

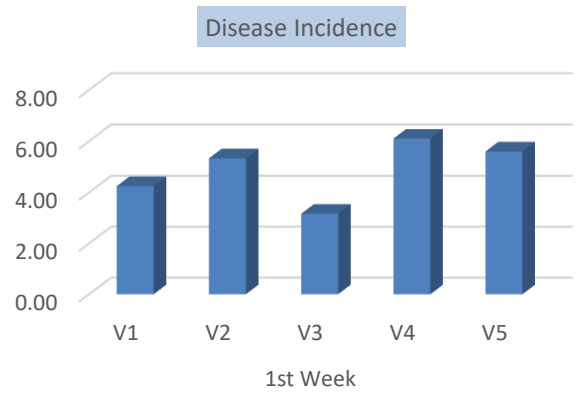

Figure 4. Disease incidence of five varieties on 1st week.

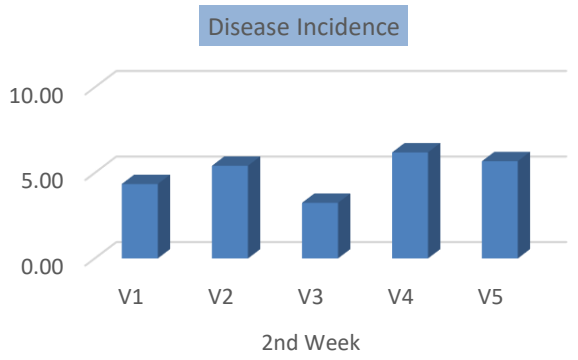

Figure 5. Disease incidence of five varieties on 2nd week.

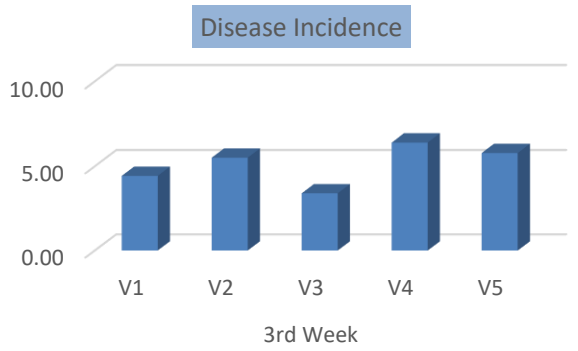

Figure 6. Disease incidence of five varieties on 3rd week.

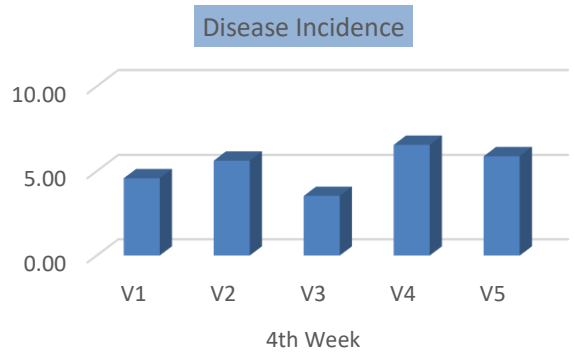

Figure 7. Disease incidence of five varieties on 4th week.

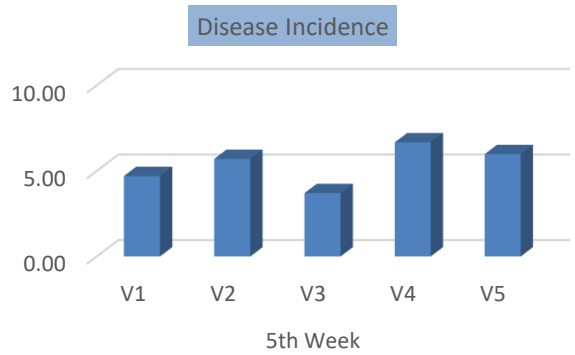

Figure 8. Disease incidence of five varieties on 5th week.

Table 6. LSD All-Pairwise Comparisons Test of Disease for Variety*Week.

Variety	Week	Mean
V1	7th	4.9500P
V1	6th	4.8500P
V1	5th	4.7000Q
V1	4th	4.5833Q
V1	3rd	4.4333R
V1	2nd	4.3500R-S
V1	1st	4.2500S
V2	7th	5.9267G-I
V2	6th	5.8333I-J
V2	5th	5.7000K-L
V2	4th	5.6200L-M
V2	3rd	5.5100M-N
V2	2nd	5.4167N-O
V2	1st	5.3333O
V3	7th	3.9167T
V3	6th	3.7833U
V3	5th	3.7000U
V3	4th	3.5333V
V3	3rd	3.4000W
V3	2nd	3.2500X
V3	1st	3.1667X
V4	7th	6.9000A
V4	6th	6.7833A-B
V4	5th	6.6667B-C

V4	4th	6.5500C
V4	3rd	6.4000D
V4	2nd	6.2000E
V4	1st	6.1000E-F
V5	7th	6.1000E-F
V5	6th	6.0333F-G
V5	5th	5.9833F-H
V5	4th	5.8800H-J
V5	3rd	5.7833J-K
V5	2nd	5.6833K-L
V5	1st	5.6000L-M

Alpha = 0.05; Standard error for comparison = 0.0664
Critical t value = 1.995; Critical value for comparison = 0.1324
Error term used: Replication*Variety*Week, 68 DF
There are 24 groups (A, B, etc.) in which the means are not significantly different from one another.

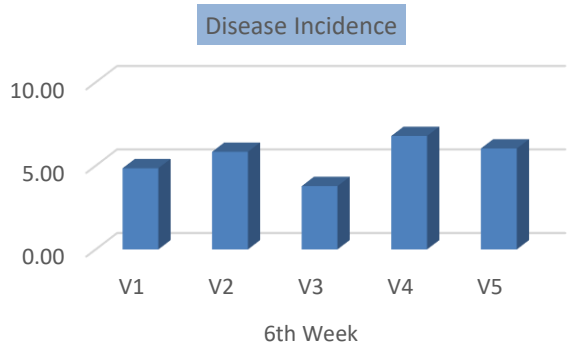

Figure 9. Disease incidence of five varieties on 6th week.

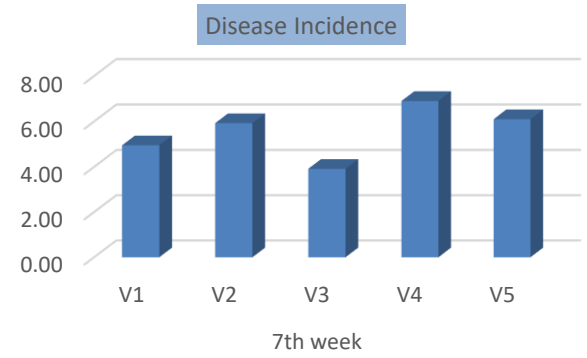

Figure 10. Disease incidence of five varieties on 7th week.

DISCUSSION

In present study, the purpose of this research, is to evaluate the severity of *S. sclerotiorum* isolates in alfalfa by sowing alfalfa cultivars in the main production area of Plant pathology, Department of Agriculture University, Faisalabad. To develop Lucerne cultivars with desirable agronomic characteristics, several hundred plant with desirable features have been needed. Breeders use several thousands of plants to initiate the breeding population because of the scarcity of resistant cultivar (and those are resistant to only one disease). To select the plants of desirable characteristics from a large number of populations, the experiment should be done under greenhouse conditions (Manrique, 1993). In this way of screening it is easy to pick the plants that are fully immune or resistant to the disease. In addition, under greenhouse condition, we can manage the plants in such a way that we are able to obtain seeds twice in the same year. On the other hand, to obtain the seeds that are resistant and tolerant characteristics against anumber of diseases under field conditions with temperate climate may take several of years. Therefore, screening is performed with the artificial infection nurseries (Kanbe et al., 1997). *Sclerotinia sclerotiorum* (Lib.) de Bary, pathogen is affecting more than 400 spoecies of plants because of its soil-borne devasting nature). As it is a soil bormne pathogen therefore it has the ability to form mycelium infection by germinating mycelium even on the surface of soil. For the penetrations of the *S. sclerotiorum* in alfalfa fields, million of the ascospores released from apotheciumfruiting bodies become the primary source of inoculum to initiate the disease (Willetts & Wong, 1980). To evaluate a number of alfalfa accessions against stem and crown rot disaes 100g of seeds are needed to evaluate them. Such screening is therefore more suitable for selecting resistant plants from cultivars. In our research, we have concluded that no such variety is completely immune to Stem and Crown Rot Disease but V3 is found to be medium resistant to resistant against the pathogen. For thorough screening against stem and crown rot disease, 100 grams of seeds per accession were employed in this research. The quantity was enough to preserve genetic variation within accessions and assure statistical significance. None of the examined cultivars showed total immunity to S. sclerotiorum, according to the results. One cultivar, known as V3, did, however, show a promising degree of durability. It was categorized as moderately resistant to resistant because it continuously showed reduced disease severity throughout replications and immediate intervals. These findings indicate that partial resistance characteristics are present and can be successfully used in breeding efforts, even though whole resistance may not be easily accessible in the current germplasm. Finding these cultivars, such as V3, is a useful initial step in creating resistant alfalfa lines via gene pyramiding or recurrent selection techniques. Additionally, under field conditions, combining resistant cultivars with additional cultural practices—like better drainage, appropriate spacing, and fungicide application—could improve disease management. The physiological and molecular characterization of resistance mechanisms in these cultivars should be the main focus of future research. This could involve marker-assisted selection to speed up breeding, biochemical testing for defense-related chemicals, and gene expression monitoring during pathogen challenge. Because of the pathogen's genetic variety and adaptability, it is necessary

to continuously monitor changes in pathogen virulence and modify breeding strategies as necessary.

***Conclusion*:** Variety V3 demonstrated strong resistance to *S. sclerotiorum*, making it a promising candidate for breeding programs. Among fungicides, Topsin M and Tiger proved most effective, significantly reducing disease severity. SAR, while moderately effective, suggests potential as a resistance inducer. An integrated approach combining resistant varieties with targeted fungicide application is recommended. These findings support the use of host resistance and chemical control as part of an integrated disease management strategy for stem and crown rot.

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