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Isolation and evaluation of culture media for mycelia growth of an emerging faba bean (*Vicia faba* L.) gall-forming disease causal agent in Ethiopia

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Abstract

The current study aimed to identify suitable preliminary culture media for the isolation of faba bean gall-forming disease causal agent and confirm by pathogenicity test. Five separate media were evaluated for isolation. Infected faba bean stem disc and the mycelial disc of a pure isolate of the test pathogen were used for the in-vitro pathogenicity test on detached leaves. Pots filled with sterilized field soil were arranged in complete random block design and sown with disease susceptible FB-26869 faba bean health seeds for pot pathogenicity test. Pycnidium of the test pathogen appears filled with mature conidia and scattered inside the infected cells. At the initial stage, it was difficult to isolate the disease causal agent on the Potato Dextrose Agar medium. The Coon's medium was found to be suitable for the preliminary isolation of the test pathogen and showed statistically significant mycelial growth (90.00 mm), while Malt Extract Agar and Tryptone Soy Agar favors a large number of conidia production at 14th day of the incubation period. The Chlamydospore and conidia of the isolates were similar to *Peyronellaea pinodella* and Phoma related species. The optimum mycelial growth of the isolates was recorded at temperature 20 ^{0}C and pH value 6.50 on both Coon's and Potato Dextrose Agar medium. No significant differences ($P > 0.05$) were recorded among leaf lesions caused by infected stem and mycelial discs. All the tested isolates exhibited similar virulence levels both in-vitro and in pot experiments. The disease incidence and severity were significantly ($P \leq 0.05$) affected by seasonal variations. The highest percent severity index (91 – 95 %) was recorded from June to September. Synthetic culture media which inhabits other fast-growing fungus favors the mycelial growth of the test pathogen. The molecular characterizations were recommended for further confirmation.

Keywords: culture media, detached leaf, faba bean, gall-forming disease, pathogenicity

1. Introduction

The faba bean (*Vicia faba* L.) locally known as "Bakela" is one of the major pulse crops commonly cultivated in the high lands of Ethiopia. Based on seed size, faba bean is known by the common names including broad bean, horse bean, tic bean, and field bean (Fatemeh *et al*., 2019). It is the leading protein source for rural people and is used to make various traditional dishes (Yitayih and Azmeraw, 2017; Etemadi *et al.*, 2019). The new emerging faba bean gall-forming disease is locally called "Kormid" threatening faba bean and causes up to complete crop failure over vast areas within a short time with disastrous economic consequences (Nigir *et al*., 2016; Bitew and Tigabie, 2016; Bekele *et al*., 2018). Faba bean gall-forming disease was first reported in July 2010 from high lands of farmers' faba bean fields around Selale and Degem, North Shoa, Oromia National Regional State of Ethiopia (Bekele *et al*., 2018; Anteneh *et al*., 2018; Alehegn *et al*., 2018).

A similar disease that attacks the stem and leaves of broad bean known as broad bean blister disease caused by *Olpidium viciae* Kusano was first reported as a new species in Japan and later in China (Yan, 2013). According to Yan (2013), the life history of *Olpidium viciae*, is parasitic on the aquatic plant *Vicia unijuga.* It has numerous but short discharging tubes and binucleate resting sporangia. *Olpidium viciae* Kusano was seldom reported throughout the world apart from Japan and China.

Depending on its field symptoms descriptions and microscopic examination, most of the Ethiopian researchers reported the fungal pathogen *Olpidium viciae* Kusano as the causative agent of faba bean gall-forming disease (Nigir *et al*., 2016; Bitew and Tigabie, 2016; Getaneh *et al*., 2018). However, these reports were not confirmed yet by colony morphology, fruiting structures, and molecular characterization. Thus, this could not be the case in Ethiopia.

Physiological and environmental factors were known to influence the growth of fungi. The causal agent of the gall-forming disease requires several specific growth conditions. Growth and sporulation are essential phases during the life of fungi, which are considerably influenced by external growth factors (Mishra and Tripathi, 2015). Among the external growth factors, nutrition was one of

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the determinants that were previously proved by several workers in fungal pathogens using different culture media sources (Adhikari *et al*., 2017; Karaoglu *et al*., 2018).

One of the most commonly exploited culture media for the growth of many fungal pathogens, Potato Dextrose Agar (PDA), was not suitable for the preliminary isolation of the fungus under study. Searching for suitable culture media for growth, conidia production, and cultural characteristic studies of the pathogen is the first step in pathological research. Synthetic culture media which supported the isolation, radial growth, and sporulation of gall-forming causal agent were not reported yet. As to our knowledge, the current study is the first attempt to find out suitable culture media and obtain the isolates of the gall-forming causal agent.

The lack of suitable culture media that support vegetative growth and sporulation of gall-forming causal agent has hindered *in-vitro* physiological and morphological studies of the test pathogen. Hence, to deal with this problem the present study was undertaken to identify the suitable culture media for mycelia growth and conidia production of the gall-forming causal agent under controlled conditions. This study mainly focused on *in-vitro* cultural practice, isolation of the causal agent, and testing the pathogenicity of the isolates both *in-vitro* and in pot experiments.

2. Materials and Methods

2.1. Sampling techniques

Simple random sampling techniques were used to address the representative of the whole faba bean plant population.

2.2. Sample collection

A total of 100 naturally infected faba bean stem/leaf with typical symptoms (small tumors) were collected from the farmers' field of Angolelana tera (50 samples), and Sululta (50 samples) districts during the 2018 main cropping season. All samples were packed in polyethylene bags and labeled with the name of the zone, district, and date of collection. The samples were transported to Mycology laboratory, College of Natural and Computational Sciences, Addis Ababa University for detection and isolation of causal agents of faba bean gall-forming diseases. It was stored at 4^0C until use.

2.3. Sample preparation for detection of the causal pathogen in the plant tissue

Infected parts of the leaves and stems were excised with a sterile scalpel. The samples of infected faba bean stem/leaf were treated with 20 ml of ethyl alcohol (80%) kept in test tubes and then dipped in boiling water until complete evaporation of ethyl alcohol to remove the chlorophyll in the plant tissues. After washing the bleached plant tissues thrice in distilled water, they were immersed in NaClO (4%) for 30 min followed by rinsing thrice with distilled water. The tissues were transferred to NaClO solution and kept at 60 °C for 15 min, followed by washing thrice in distilled water (Caiazzo *et al*., 2006). The tissue was stained with lactophenol cotton blue and examined under a 40 x objective lens of the compound light microscope (MAX BINO BELGIUM).

2.4. Preliminary isolation and purification of the gall-forming causal agent

Initially, the small pieces (1 cm) of infected faba leaves were excised and surface-sterilized by dipping them in 2% sodium hypochlorite for 5 minutes, followed by washing three times with sterile distilled water for 2 minutes. The sterilized fragments of infected faba bean leaves were inoculated on 90 mm Petri dishes containing sterilized Coon's medium (4 g/l maltose, 2 g/l KNO3, 1.20 g/l MgSO4.7H2O, 2.68 g/l KH2PO4, and 20 g/l agar) (Adnan *et al*., 2017) amended with chloramphenicol. A 0.1 ml of the last washes was spread plated on Coon's medium as a control to check the quality of surface sterilization. The inoculated medium was incubated at 23 ± 2 ^{0}C for 14 days. The actively growing parts of the isolates were purified on the Coons medium using single hyphal tip techniques suggested by Ahmed and El-Fiki (2017).

2.5. Evaluation of culture media for mycelial growth and conidia production

To find out the medium that best suits for the mycelia growth, conidia production, and other cultural characteristics of gall-forming disease causal agent, five culture media were compared, including Coon's medium (CN), Potato Dextrose Agar (PDA) (Oxoid), Malt Extract Agar (MEA) (Oxoid), Tryptone Soy Agar (TSA) (Oxoid), and Water Agar (WA) in solid states. Pure mycelia Coon's agar plug measuring 4 mm diameter were taken from the actively growing margin of 12 day-old pure culture using a sterile cork borer and placed upside down in the center of each medium in a 90 mm Petri dish. The Petri plates were immediately sealed with parafilm with four replications. All the plates were incubated at 23 ± 2°C for 14 days and all the activities were done under aseptic conditions inside the laminar flow cabinet. Visual observations concerning colony growth were made starting from the fourth day after incubation. The mycelia diameter of the isolates on different media was measured and compared quantitatively, whereas the conidia productions were recorded qualitatively (Smita and Dhutraj, 2017).

2.6. Morphological characterization

2.6.1. Macroscopic morphology

The cultural morphology studies of the isolates were performed on MEA, WA, PDA, Coon's Medium, and TSA following the standardized procedure. The colony diameters (mm) were measured starting from the fourth day after incubation at every two days interval until the mycelium fully covered the Petri dish. Colony morphology, shape, and other cultural characteristics were observed with the naked eye throughout the incubation period and characterized at full plate colony growth on 14 days of incubation. Colony growth was measured in two days intervals. Colony colors and texture were rated 14 days after incubation (Ahmed and El-Fiki, 2017).

2.6.2. Microscopic morphology

Pure fungal isolates were mounted in distilled water with a scalpel blade to study the structure of Pycnidia, chlamydospores, and conidia (Ahmadpour *et al*., 2017). The microscopic examinations were carried with the 40X objective lens of the compound light microscope (MAX BINO BELGIUM). Macroscopic and microscopic morphological characters were used to compare the fungal

isolates with the assistance of current mycological literature (Johnston *et al*., 2017).

2.7. Effect of temperature and pH on growth of gall-forming causal agent

2.7.1. Effect of temperature

The ability of faba bean gall-forming diseases causing pathogen to grow at restrictive temperature was assessed by growing the cultures on PDA and Coon's medium and incubating at 20, 23, and 25 °C until full growth of the mycelia in 90 mm plate followed by You *et al*. (2016). For measuring the diameter of mycelia growth rate, the fungal isolate was inoculated in triplicates at the center of the medium. The inoculum was aseptically punched with a cork borer in the form of four mm mycelial discs from the seven days old margin of colonies. The plates were incubated at different temperatures and the diameter of the mycelial growth was measured (in mm) every two days interval up to full growth of mycelia in the plates.

2.7.2. Effect of pH

The pH values 6.50, 7.00, and 7.50 within the range of soil pH of the study sites were used for the study of the effect of pH on the growth rate of faba bean gall-forming disease-causing pathogen as described by You *et al*. (2016). PDA and Coon's medium were prepared in triplicates and its pH was adjusted by adding HCl and NaOH before autoclaving. A four mm disc from the margin of seven days old culture of the gall-forming pure isolate was inoculated on the plates containing PDA and Coon's medium. The inoculated plates were incubated at the temperature mentioned above. Measurements of the diameter of the mycelia growth were recorded as described above.

2.8. Detached leaf in-vitro pathogenicity test

This was done by two experiments. Experiment 1 was conducted to determine the pathogenicity of infected faba bean stems/leaf with typical gall disease symptoms. Experiment 2 was conducted to determine the pathogenicity of faba bean gall-forming diseases causing suspected isolates. In-vitro pathogenicity of both the infected disc of stem/leaf and suspected isolates was done on fresh leaflets of faba bean as stated by Kayim *et al*. (2018).

2.8.1. Preparation of leaflets

Faba bean seeds (FB-26869) were sown in pots at the College of Natural and Computational Sciences, Addis Ababa University greenhouse on December 4, 2018. Leaves were collected from 60 days old seedlings. Leaflets were surface-disinfected by immersion in dilute sodium hypochlorite (2% active chlorine) for 1 min, rinsed three times with sterile distilled water, and placed under an air stream to remove excess water followed by Ermias *et al*. (2013).

2.8.2. Inoculums preparation and inoculation

The infected and healthy portions of faba bean stem were separately cut into four mm discs by corks borers. Both infected and healthy faba bean stem discs were surface sterilized with 2 % Sodium hypochlorite. Similarly, 14 days old culture of agar plugs containing five different faba bean gall-forming diseases causing suspected isolates were removed using a cork borer. A four mm disc of infected faba bean stems and the agar plugs were inoculated at the center of pre-prepared leaflets separately followed by Kayim *et al*. (2018). The health faba bean stems disc and isolates free agar plugs were placed on leaflets as control. All were done in triplicates. Then the inoculated leaflets were placed face-up on the filter paper impregnated with sterile distilled water in the sterilized Petri plates to serve as a moist chamber. All cultures were kept at room temperature ($20 \pm 3\ ^0$C) for five days. The length and width diameters of the leaves lesion formed around each stem disc and agar plugs were measured separately. The lesions of artificially infected leaflets were examined by microscope (MAX BINO BELGIUM) and confirmed as the original inoculated isolates and natural infections.

2.9. Pot experiment pathogenicity test

2.9.1. Experimental design and details

The pot experiment pathogenicity test was conducted three times at the College of Natural and Computational Sciences, Addis Ababa University greenhouse. The first, second, and third experiment was carried out during December 2018 – February 2019, March 2019 – May 2019, and June 2019 – September 2019, respectively. A total of 18 pots (20 cm diameter) were surface sterilized by using 70 % alcohol and arranged in Complete Random Block Design (CRBD) in three blocks (Zakawa *et al*., 2018). A 2 kg of 2 mm sized sieved autoclaved field soils were added to each pot. The pots were kept for one week and irrigated regularly. The most susceptible faba bean accession (FB-26869) to gall-forming diseases causing pathogen was used for the pathogenicity test.

2.9.2. Inoculum preparation and inoculation of the pathogen

Based upon the in-vitro antigenicity test result, four mm discs of five selected faba bean gall-forming diseases causal agent suspected isolates were removed by using sterilized cork borers. Three discs of each isolate were separately inoculated in a 250 ml flask containing 100 ml Coon's broth and incubated at 23 ± 2^0C for 14 days. Twelve faba bean seeds (FB-26869) were impregnated with 30 ml of conidia suspensions (10^5 spores/ml) of each isolates separately in a 250 ml flask and incubated at $23 \pm 2\ ^0$C for 48 hr. The level of conidia suspensions was adjusted by using a hemocytometer. Four faba bean seeds impregnated with the suspected isolates were sown in each pot as stated by Khaledi and Taheri (2016).

Besides, for the mass production of the isolates, 10 ml of each suspension (10^5 spores/ml) of suspected isolates were separately added to 100 g sterilized ground faba bean stems and incubated at $23 \pm 2\ ^0$C for 21 days (Karaoglu *et al*., 2018). Fourteen days after the emergence of seedlings, each pot was inoculated with 10 g of the corresponding mass-produced faba bean gall-forming causal agent suspected isolates to increase the inoculums sources. Twelve faba bean seeds impregnated with sterile distilled water served as control. Three replications were maintained. The pathogenicity of each isolate was evaluated through observation and recording the disease incidence and severity per seedlings 30 days after the seedling emergence. The pathogenic isolates were re-isolated from the newly infected faba bean seedling stems and leaves. Then, compared and confirmed with the

original inoculated isolates by microscopic morphological characterization.

2.9.3. Disease data

Terminal disease incidence and present severity index were recorded on plant bases to evaluate the faba bean gall-forming disease causal agent. Percent disease incidence computed over the number of diseased plants from the total number of inspected plants. The disease percent severity index (PSI) was calculated using a 0–9 scale to determine the area of the affected plant part, according to Aghajani *et al*. (2009), with little modifications.

$$PSI = \frac{(a + 3b + 5c + 7d + 9e)}{9(a + b + c + d + e)} \times 100$$

Where, 9 = highest rating value, a= number of plants in class 1, b = number of plants in class 3, c = number of plants in class 5, d = number of plants in class 7, e = number of plants in class 9.

2.10. Statistical data analysis

The data were subjected to SPSS statistical software version 20 and one way ANOVA. The effect of culture media on the mycelial growth of faba bean gall-forming disease causal agent was compared using the least significant difference (LSD) at a 5% probability level ($P \leq 0.05$). The slopes were used as measures of mycelia radial growth rates (mm day^{-1}) for each culture medium source treatment (Smita and Dhutraj, 2017).

3. Results

3.1. Detection of faba bean gall-forming disease causal agent inside the infected cells

Light microscopy observations of infected faba bean stem with the gall-forming disease were presented in Figure 1. The infected cells appeared plasmolyzed and disorganized. Pycnidia of the gall-forming causal agent scattered inside the infected faba bean cells. It appears filled with a large number of mature conidia.

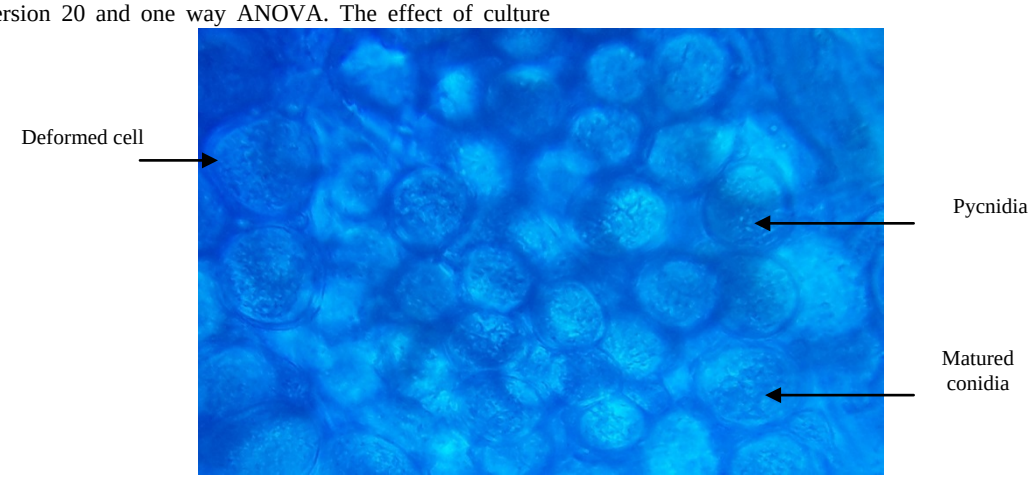

Figure 1. Light microscopy observation of the infected faba bean stem cell stained with lactophenol cotton blue

3.2. Preliminary isolation and purification of the isolates

Different synthetic culture media were tested for isolation of faba bean gall-forming pathogen. It was difficult to isolate the pathogen by using potato dextrose agar from the infected plant parts at the initial stage. However, Coon's medium was very important for the isolation and purification of the pathogen at the preliminary stage. The pure isolates can grow on potato dextrose agar. Thus, potato dextrose agar slant could be used for the storage of the pure isolates at 4 ^{0}C for further use.

3.3. Evaluation of culture media for mycelia growth

The mycelial growth rate of the test pathogen was affected by variation in culture media sources. The tested culture media supported the growth of the pure isolates of faba bean gall-forming disease causal agent to various degrees and significantly ($P \leq 0.05$) affected mycelia growth of the pathogen at the end of the growth period (Table 1). The mean colony diameter measured with the entire test media ranged from 70.00 (WA) to 90.00 mm (Coon's medium) at 14 days after incubation. Among the tested culture media, Coon's medium (90.00 mm) showed statistically higher mycelial growth followed by PDA (84.00 mm) than others. Coon's medium was found to be more suitable for vegetative growth than the other culture media.

Table 1. *In-vitro* evaluation of different growth culture media on colony growth (mm), growth rate (mm/day) of faba bean gall-forming causal agent incubated at 23 ± 2°C

Medium type	Mean diameter ± SD of colony growth (mm) after different incubation periods (hr)							Radial growth rate (mm/day)	R^2 (%)
	48	96	144	192	240	288	336		
MEA	$12^a \pm 2$	$18^a \pm 5$	$24^a \pm 0$	$36^a \pm 2$	$52^a \pm 2$	$70^a \pm 1$	$80^c \pm 1$	0.12^a	96
WA	$12^a \pm 1$	$16^a \pm 4$	$20^a \pm 2$	$36^a \pm 3$	$48^a \pm 2$	$64^a \pm 2$	$70^b \pm 2$	0.89^b	99
PDA	$16^a \pm 1$	$24^b \pm 3$	$36^b \pm 4$	$50^b \pm 1$	$64^b \pm 5$	$76^b \pm 1$	$84^d \pm 2$	1.08^c	96
Coon's	$16^a \pm 3$	$24^b \pm 2$	$40^b \pm 3$	$52^b \pm 2$	$68^b \pm 6$	$84^b \pm 2$	$90^a \pm 3$	1.10^c	99
TSA	$12^a \pm 4$	$16^a \pm 1$	$28^a \pm 2$	$36^a \pm 1$	$50^a \pm 4$	$68^a \pm 2$	$76^e \pm 2$	0.85^b	98
Average	$13^a \pm 1$	$19^a \pm 2$	$29^a \pm 4$	$41^a \pm 5$	$56^a \pm 4$	$72^a \pm 4$	$80^c \pm 4$	$0.81^b \pm 0.4$	97.6 ± 2
CV (%)	16	21	28	22	16	11	10	50	2

Means of mycelial growth followed by a different letter (s) in the same column are significantly ($p \leq 0.05$) different, MEA: Malt Extract Agar, WA: Water Agar, PDA: Potato Dextrose Agar, TSA: Tryptone Soy Agar, R^2: Coefficient of determination, SD: Standard deviation

3.4. Macroscopic morphological characteristics

The test pathogen exhibited a wide range of colony characteristics about shape, and color on MEA, WA, PDA, Coon's medium, and TSA on 14th day of incubation period (Figure 2). Mycelia growth patterns included both light and dense radial extending mycelium. Radial mycelia growth was consistently a characteristic feature of the fungus on all media sources. Both the reverse and the front sides of the colony exhibited various colors and shapes on different culture media (Table 2 and Figure 2). Light mycelia, brown-black ring center followed by white ring regular margins were recorded on the reverse side, whereas white regular double rings were recorded on the front side of Coon's medium.

On the other hand, dense mycelia, brown color at center with regular colony margins were recorded on the reverse side of the PDA medium. The black center followed by the white and regular brown rings was recorded on the front side of the PDA medium. Light mycelia were recorded on MEA, Coon's, and TSA medium. Very light and irregular shaped black center mycelia were recorded on WA. The microscopy of a colony grown on different media showed that large numbers of conidia were recorded on MEA and TSA (Table 2 and Figure 2).

Table 2. Cultural characteristics and conidial production of gall-forming disease causal agents on 14 days of incubation at 23 ± 2 ^{0}C

Medium	Conidia	Cultural characteristics	
		Reverse side color and shape	Front side color and shape
MEA	+ + +	white irregular shape at the center greenish-brown irregular shape at the middle white irregular margin	white irregular shape at the center black with a double ring in the middle white irregular margin
WA	-	very light mycelia with irregular black color at the center	very light mycelia with irregular black color at the center
PDA	+	brown at the center and middle with a white regular margin	black center followed by a white and brown ring with a white ring
Coon's	+ +	a brown black ring at the center followed by a regular white ring	white double-ring followed by white regular ring
TSA	+ + +	yellowish white with regular margin	white with regular margin

- = no condia, + = poor, ++ = fair, and +++ = good

MEA: Malt Extract Agar, WA: Water Agar, PDA: Potato Dextrose Agar, TAS: Triptone Soy Aga

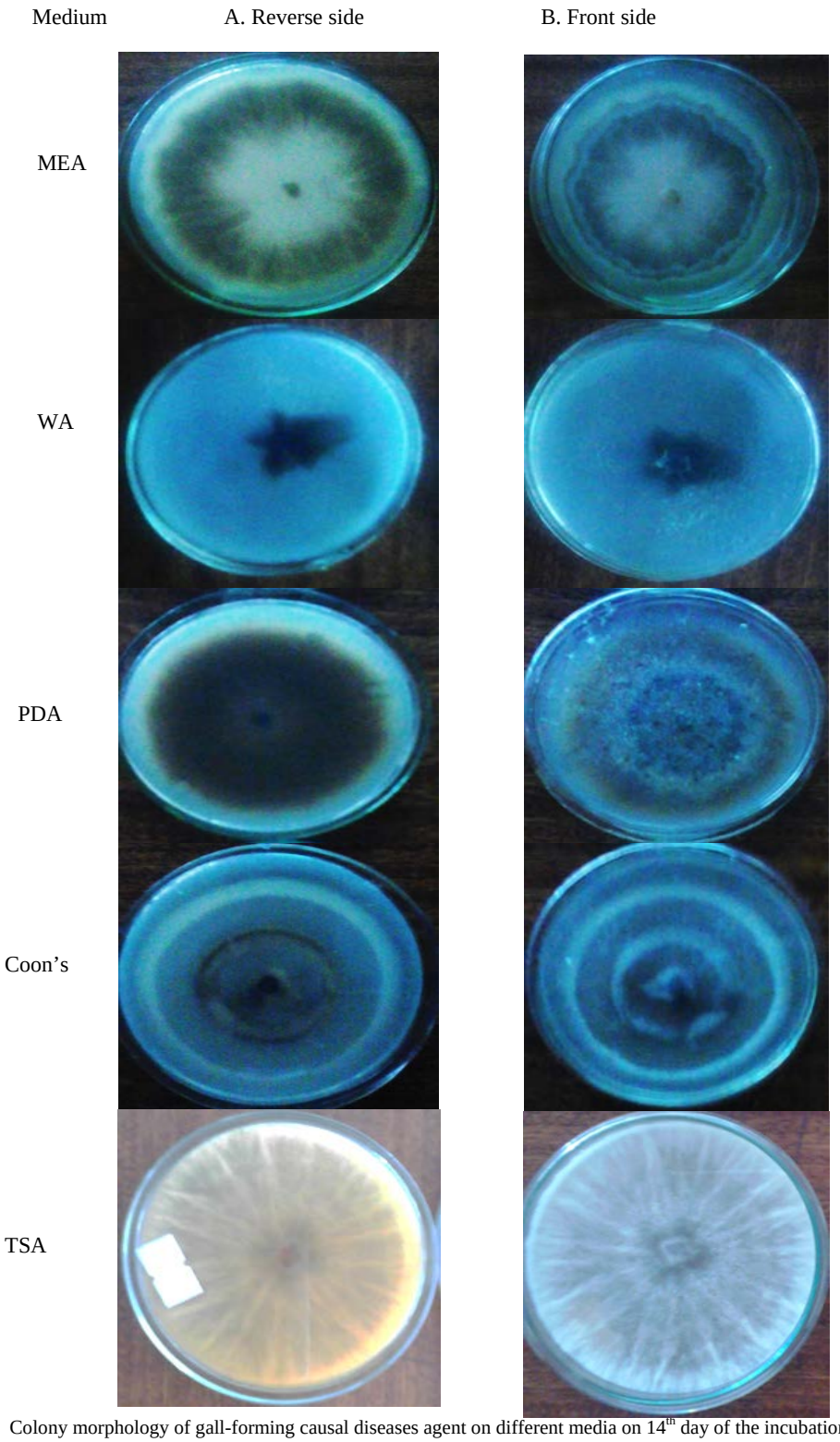

Figure 2. Colony morphology of gall-forming causal diseases agent on different media on 14th day of the incubation period at 23 ± 2 ^{0}C. MEA: Malt Extract Agar, WA: Water Agar, PDA: Potato Dextrose Agar, TSA: Tryptone Soy Agar

3.5. Microscopic morphological characteristics

The microscopic structures pycnidia, chlamydospore, and conidia were commonly observed in the 14 days old culture of faba bean gall-forming disease-causing isolates. Mature pycnidium was often observed on the tip of mycelium (Figure 3 A). Chlamydospore was observed frequently in a chain and separately on mycelia intercalary and terminally (Figure 3 C). Sub cylindrical to the narrow ellipsoid shape of conidia with two nuclei were observed frequently (Figure 3 B) under light microscope.

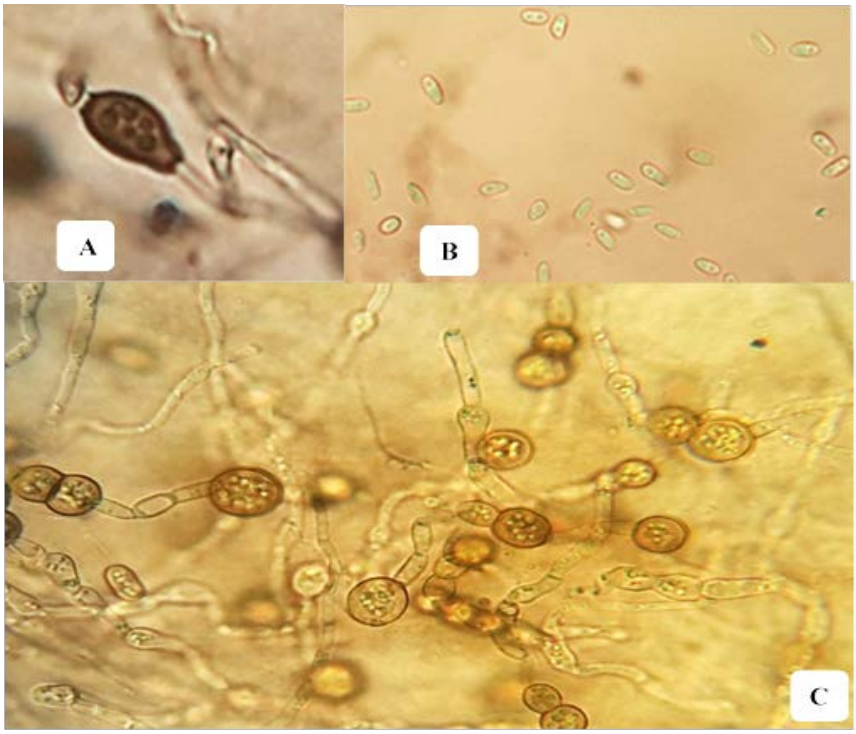

Figure 3. The microscopic structure of gall-forming disease causal agent stained with sterilized distilled water, A: Pycnidia, B: Condia, C: Chlamydospore

3.6. Effect of temperature and pH on the in-vitro growth of gall-forming causal agent

Incubation temperature significantly ($P \leq 0.05$) affected the mycelia growth of the gall-forming causal agent. As the temperature increased from 20 ^{0}C to 25 ^{0}C, the mycelia growth of the isolates were significantly decreased. On the other hand, the current evaluated pH values had not significantly influenced the mycelial growth. The maximum mycelial growth (86 mm) was recorded at temperature 20 ^{0}C and pH value 6.50, whereas the minimum (72.67 mm) was recorded at temperature 25 ^{0}C and pH value 7.00 on PDA medium (Figure 4). At temperature 20 ^{0}C and pH value 6.5, 88.67 mm mycelia diameter of the gall-forming isolates was recorded on Coon's medium (Figure 5). Thus, Coon's medium was more suitable for the growth of gall-forming pathogen than the PDA medium. The optimum growth of the isolates was recorded on temperature 20 ^{0}C and pH value 6.50 on both mediums.

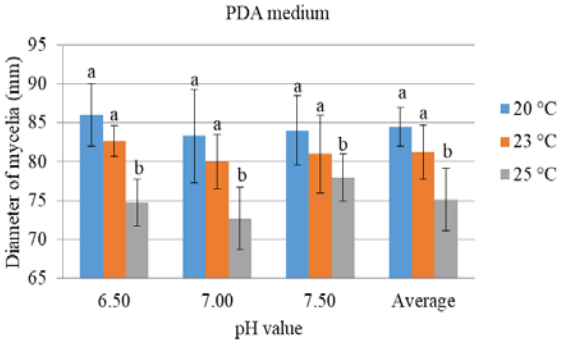

Figure 4. The effect of temperature and pH on the mycelia growth of gall-forming pathogen on PDA medium at 14th day of the incubation period there was no significant difference between bar graphs labeled with the same letters, a vertical line on the graphs indicates the standard deviation

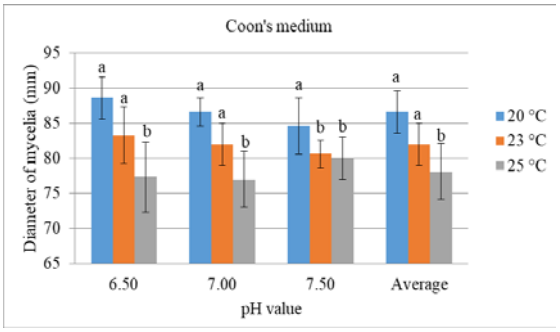

Figure 5. The effect of temperature and pH on the mycelial growth of gall-forming pathogen on Coon's medium at 14th day of the incubation period

there was no significant difference between bar graphs labeled with the same letters, a line on the graphs indicates the standard deviation

3.7. Detached leaves in-vitro pathogenicity test of gall-forming disease causal agent

A significant difference ($P \leq 0.05$) were observed between the average length diameter of the leaf lesion, whereas there was no significant difference ($P > 0.05$) in the average width diameter of the leaf lesion caused by infected stem disc (Experiment 1) and mycelia disc of gall-forming disease-causing isolates (Experiment 2). Among infected stem disc inoculated leaves, the maximum lesion length diameter (28 mm) and width diameter (17.33 mm) were recorded by disc 4. But, the minimum lesion length diameter (6.66 mm) and width diameter (7.33 mm) recorded by infected stem disc 5. On the other hand, the maximum lesion length diameter (29.67 mm) were recorded by the mycelia disc of isolate 6, whereas the maximum lesion width diameter (25 mm) was recorded by the mycelia disc of isolate 4. Leaflets inoculated with health stem disc and mycelia free agar plug did not develop infection symptoms (Table 3 and Figure 6).

Table 3. Average leaf lesions in detached leaf pathogenicity test

Inoculum sources	LD of the leaf lesions (mm) ± SD	WD of the leaf lesions (mm) ± SD	LD/WD leaf lesions
Experiment 1			
Infected stem disc 1	14.00^{a} ± 0.00	13.33^{d} ±1.15	1.05
Infected stem disc 2	15.33^{a} ± 1.15	15.33^{d} ± 1.15	1.00
Infected stem disc 3	22.00^{e} ± 15.62	12.33^{d} ± 7.37	1.78
Infected stem disc 4	28.00^{d} ± 21.37	17.33^{d} ± 11.54	1.62
Infected stem disc 5	6.66^{b} ± 3.05	7.33^{e} ± 3.05	0.91
Infected stem disc 6	20.67^{e} ± 17.01	10.00^{e} ± 6.00	2.07
Infected stem disc 7	21.67^{e} ± 21.08	12.67^{d} ± 8.08	1.71
Infected stem disc 8	24.67^{d} ± 18.58	13.33^{d} ± 8.32	1.85
Control	0.00^{f}	0.00^{f}	
Average	19.13^{e} ± 6.79	12.71^{d} ± 3.06	1.51 ± 0.44
CV (%)	35.49	24.07	29.13
LSD (P = 0.05)	0.73	0.74	
Experiment 2			
Mycelia disc of isolate 1	6.00^{b} ± 0.00	6.00^{a} ± 0.00	1.00
Mycelia disc of isolate 2	14.00^{a} ± 6.00	13.33^{d} ± 7.02	1.05
Mycelia disc of isolate 3	10.00^{g} ± 6.00	8.00^{a} ± 3.46	1.25
Mycelia disc of isolate 4	16.00^{a} ± 5.29	25.00^{b} ± 13.23	0.64
Mycelia disc of isolate 5	9.33^{g} ± 4.62	9.33^{c} ± 5.03	1.00
Mycelia disc of isolate 6	29.67^{d} ± 22.37	16.00^{d} ± 10.58	1.85
Mycelia disc of isolate 7	8.00^{b} ± 6.93	6.00^{a} ± 3.46	1.33
Mycelia disc of isolate 8	22.67^{e} ± 16.29	12.00^{d} ± 6.93	1.89
Control	0.00^{f}	0.00^{f}	
Average	14.46^{a} ± 8.11	11.96^{d} ± 6.35	1.25 ± 0.43
CV (%)	56.12	53.07	34.65
LSD (P = 0.05)	0.18	0.08	

LD: length diameter of the leaf lesions, WD: width diameter of the leaf lesions, SD: standard deviation

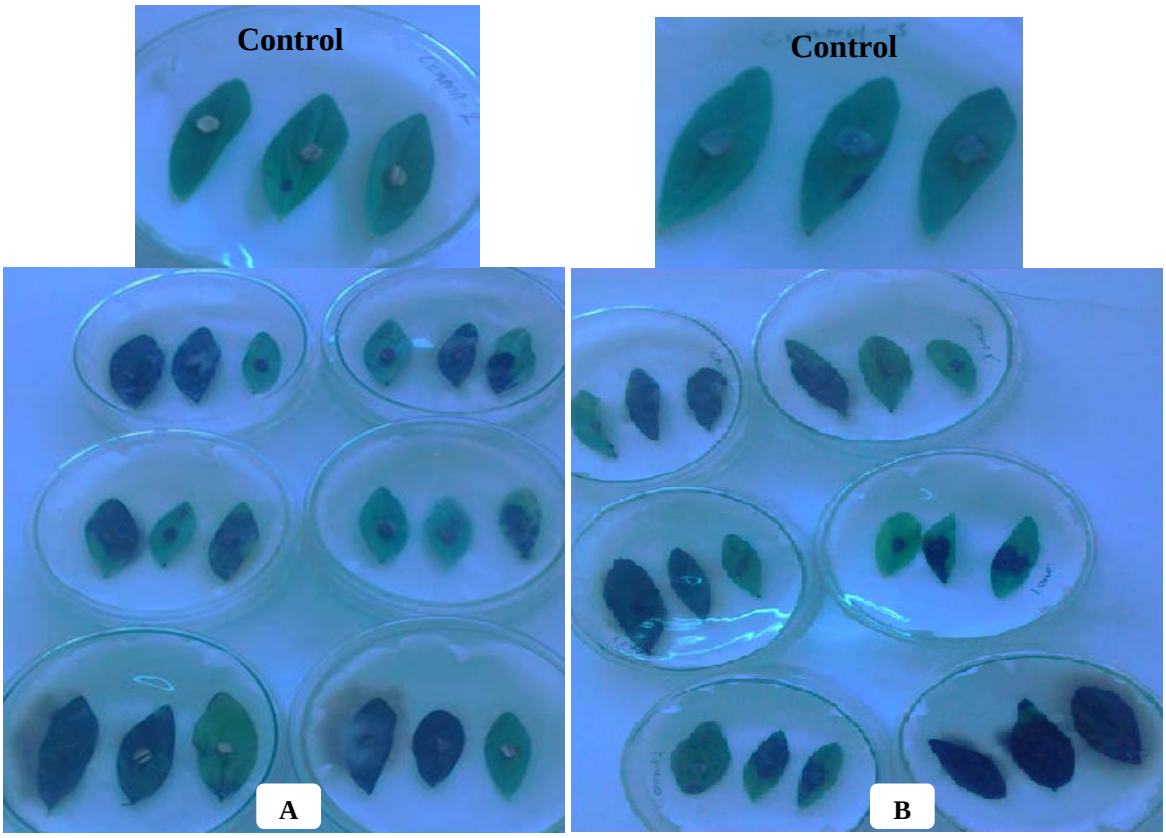

Figure 6. *In-vitro* pathogenicity test on detached leaves, A. Experiment 1: infected faba bean stem disc B. Experiment 2: Mycelia disc of gall-forming disease-causing isolates

3.8. Pot experiment pathogenicity test of gall-forming disease causal agent

It has been noted that gall-forming disease symptoms appeared after thirty days of plant emergence and continues until flowering. The symptoms appear on leaves and stems. Ten to 25 chlorotic small galls formed on a single leaf. The gall progressively enlarged and became light brown, circular, or elliptical rough spots (Figure 7 C).

At the later stage, it turns to black or brown, the tissues decay, and a few galls break to form necrotic areas (Figure 7 D). Galls often coalesce adjacently to form huge galls, resulting in rolling up (cupping) and finally kill the infected leaves (Figure 7 E). Faba bean seedlings not inoculated with the pathogens (control) did not develop infection symptoms (Figure 7 B).

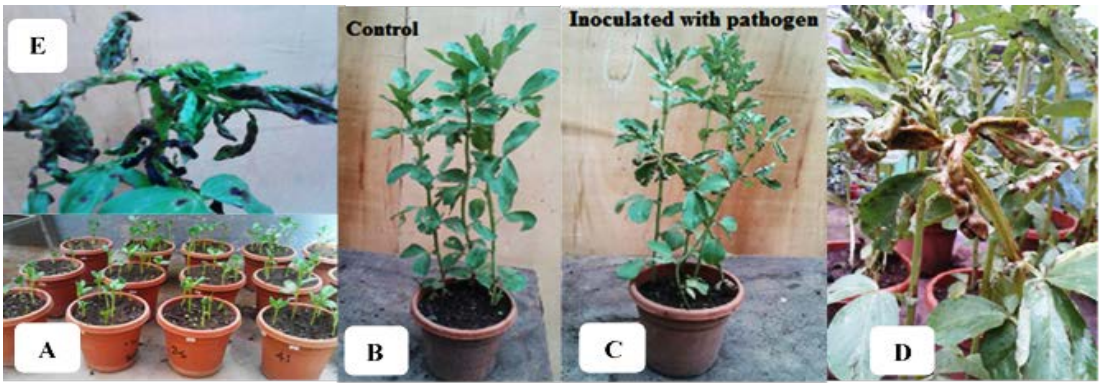

Figure 7. Faba bean gall-forming disease causal agent pathogenicity test in pot experiment during June 2019 – September 2019; A: 14 days faba bean seedlings, B: none inoculated with a pathogen, C: inoculated with pathogen D: faba bean gall-forming disease symptoms, E: faba bean leaves dead by gall-forming disease.

3.9. Percent disease incidence and severity index

During December 2018 – February 2019 (Experiment 1) and March 2019 – May 2019 (Experiment 2) no isolates caused disease incidence on faba bean seedlings (Table 4), whereas, during June 2019 – September 2019 (Experiment 3), both isolates caused faba bean gall-forming disease symptoms on all (100%) faba bean seedlings expect in control. The highest significant ($P \leq 0.05$) disease incidence and severity were recorded on experiment 3 than experiment 2, and experiment 1. There was no significant difference ($P \geq 0.05$) both in disease incidence (100 %) and PSI (91 – 95 %) among isolates in experiment 3 (Table 4).

Table 4. Disease incidence and severity index of faba bean gall-forming disease-causing isolates in a pot experiment

Control and disease causing isolates	Experiment 1 (December 2018 – February 2019)		Experiment 2 (March 2019 – May 2019)		Experiment 3 (June 2019 – September 2019)	
	Incidence (%)	PSI (%)	Incidence (%)	PSI (%)	Incidence (%)	PSI (%)
Control	0	0	0	0	0	0
Isolate 2 (AAUO2)	0	0	0	0	100	91
Isolate 3 (AAUO3)	0	0	0	0	100	93
Isolate 4 (AAUO4)	0	0	0	0	100	95
Isolate 6 (AAUO6)	0	0	0	0	100	94
Isolate 8 (AAUO8)	0	0	0	0	100	95
Average	0	0	0	0	100	93.60

PSI: a percent severity index

4. Discussion

An emerging faba bean gall-forming disease is one of the most devastating fungal diseases infecting and constraining faba bean cultivation in Ethiopia (Bekele *et al*., 2018). Recently, the disease is expanding in faba bean growing areas of the country, especially in altitudes above 2400 m above sea level (a. s. l.) (Anteneh *et al.*, 2018). The pathogen-infected and parasitized in the epidermal cells of the faba bean leaf and stem. The host responded quickly to the invasion and then it induces the gall symptom (Figure 1).

In this study, culture media strongly influenced the growth and conidial productions of faba bean gall-forming disease causal agent. Several studies also found similar results concerning the effect of culture media on growth, sporulation, and other cultural characteristics of various types of fungi (Koley and Mahapatra, 2015; Mishra and Tripathi, 2015). Coon's culture medium supported the slow grower faba bean gall-forming causal agent. It was found to be the most suitable for the preliminary isolation and favors the mycelial growth of the fungus under study. This could be attributed to the low glucose content of the medium and its ability to inhibit other competing fast grower fungus.

Malt extract agar and Tryptone soy agar resulted in good and abundant, whereas the rest tested culture media showed no (Water agar), poor (Potato dextrose agar), and fair (Coon's medium) in conidia production. Good conidia production of the media could be attributed to the low sugar content of the medium. Similarly, another study by Koley and Mahapatra (2015) pointed out that Oat Meal Agar (OMA) supported better sporulation of *A. solani* than PDA due to lower sugar content. In contrast to the current study, Smita and Dhutraj (2017) indicated that PDA and other culture media having good sugar content allowed the best mycelial growth of *A. solani*.

This study found that the colony characteristics of gall-forming disease causal isolates on various culture media were similar to Phoma related species. Phoma related species are associated with disease on many hosts, including legumes (Ahmadpour *et al*., 2017). Besides, the

diagnosis of microscopic structures showed that the chlamydospore (Figure 3 C) and conidia (Figure 3 B) of the isolates were similar to *Peyronellaea pinodella*. *Peyronellaea pinodella* is associated with a black stem (summer back stem) of clover and peas (Johnston *et al*., 2017).

At the same time, temperature significantly influenced the mycelia growth rate. Low temperature (20 ^{0}C) was preferred by gall-forming disease causal isolates among the tested temperature. However, 6.5 – 7.5 pH values had no significant influence on the mycelia growth of the isolates. In line with our study, Zehra *et al*. (2017) reported that temperature has a great influence on radial growth and sporulation of the fungus. The pH values of the nutrient medium determine mineral availability and influence metabolic rates of the fungus (Poosapati *et al*., 2014).

The artificially inoculated test pathogen on detached leaflets produced progressive lesions that expanded from the inoculation point through the leaf. The virulence of both infected faba bean stem disc and agar plug of each isolate were consistent (Table 3 and Figure 6). This result coincides with the report of Kayım *et al*. (2018) and Ahmadpour *et al*. (2017) who reported the effect of leaf spot disease on faba bean detached leaves, and the pathogenicity of *Didymella microchlamydospora* causing stem necrosis of *Morus nigra* in Iran, respectively. Detached leaf tests could be interesting because they can be performed rapidly, and results can be measured in a shorter period (5 – 7 days). Light microscopy observations demonstrated that the test pathogen can penetrate, colonize, and infect the leaf tissues since progressive tissue deterioration and cell plasmolysis were observed five days after pathogen inoculation. These changes in cell structure can be compared with those reported by Ahmadpour *et al*. (2017) and Johnston *et al*. (2017).

In a previous study, Bitew and Tigabie (2016) reported that at the initial stage small chlorotic galls are formed on faba bean leaves, and then progressively enlarge to become light brown, circular, or elliptical rough spots. The small tumor-like galls are formed adjacently to form huge galls, resulting in rolling up and abnormal growth of leaves. The current study also found similar disease symptoms only during June 2019 – September 2019 (experiment 3) in the pot experiment pathogenicity test. This showed that seasonal variation significantly affected the occurrence of disease symptoms on faba bean seedlings. The faba bean gall-forming disease incidence, conidia germination, and virulence of the fungus understudy were favored by the low temperature and high humidity of the cropping season. This result coincides with Yan (2013) findings who reported on blister disease of broad bean in China.

5. Conclusion and Recommendations

Coon's culture medium was suitable for the preliminary isolation and mycelia growth of faba bean gall-forming disease-causing isolates, whereas Malt extract agar and Tryptone soy agar supported good conidia formation. Seasonal variations significantly affected the incidence of faba bean gall-forming disease. Particularly, low temperature and high humidity favor disease incidence and severity. The macroscopic colony morphology and microscopic morphological structures of the test pathogen were similar to *Peyronellaea pinodella* and Phoma related species. Therefore, the whole genome analysis and molecular characterization should be conducted for further confirmation of the disease causal agent.

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