SUCCESSFUL ISOLATION, GROWTH AND SPORULATION OF *UROMYCES FABAE* IN AXENIC CULTURES

BY

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ABSTRACT

For the first time in the world using the usual technique for isolating plant fungal pathogens, successful growth and sporulation of *Uromyces fabae* (Pers.) De Bary, the causal of broad bean rust, was achieved axenically on different dilutions (strengths) of a modified MURASHIGE & SKOOG's (MS-) tissue culture medium. The linear growth of *Uromyces fabae* was more pronounced on media containing pollen grains only and/or inositol. Regardless media strength, it was found that the MS- medium containing pollen grains at the rate of 10 g/l. was the best for producing uredospores. However, the total number of uredospores was reduced proportionally by increasing dilution of media strength. Few teleutospores were also observed on all tested media. Effect of the tested medium and its components on characters of the resultant uredospores was also included. Under conditions of the present work, uredospores of *Uromyces fabae* from axenic cultures on MS-medium could not germinate on surfaces of inoculated broad bean leaves and reasonably completely failed to cause any symptoms of rust disease.

INTRODUCTION

Axenic culture has been defined by DOUGHERTY (1953) as referring specifically to the growth of one organism free of all others. Obligate parasites therefore, are those organisms which can't be cultured on nonliving substrates and drive their nutrition from another living organism and can not be grown in an axenic culture. YARWOOD (1956) stated that culturing of parasites on non-living substrates greatly aids our understanding of their nutrition and physiology, but it was not clear at present that culturing of obligate parasites of plants would aid in their control. The difference between obligate and non-obligate parasites was well emphasized by their response to growth on potato dextrose agar. Most non-obligate parasites made more growth on potato dextrose agar than on water, while most obligate parasites made more growth on water or water agar than on potato dextrose agar. Most nutrients which favor growth of non-obligate parasites could not even enhance spore germination of most obligate parasites (DUGGAR, 1909). WILLIAMS et al. (1967) noted that the addition of 0.1% Evan`s peptone to the medium containing yeast extract enhanced vegetative growth from uredospores of wheat stem rust (*Puccinia graminis* f.sp. *tritici*). Uredospores and teleutospores were formed on stromata. The pathogenicity of saprophytic cultures was demonstrated by several ways. Firstly, by inserting mycelium free of agar (from 20-30 days old cultures) under the epidermis of the first leaf of wheat plants and the inoculated area was covered with parafilm then plants were placed in moist chamber at 17°C in the light. Secondly, by removing the lower epidermis from surface sterilized segments (3-4 cm) of noninfected leaves of Little Club wheat and placing them on cultures with the exposed mesophyll in contact with the vegetative mycelium and incubated in a saturated atmosphere at 17°C. BUSHNELL (1968)

failed in his first attempts to apply the methods of Williams et al. (1967) but he found that the medium which supported in vitro growth of an Australian isolate of *Puccinia graminis* f.sp. *tritici* contained 0.1% Evan`s peptone, 3% glucose, Czapek`s mineral salts, and 2% agar. Tiwari and Arya, (1969) reported that the the downy mildew fungus *Sclerospora graminicola* can grew from the infected callus tissue from the hypocotyles of *Pennisetum typhoides* and cover the surface of the medium. The medium used was modification of White's basal mineral salts and containing the following (per 1): glucose, 20 g; agar, 5 g; casein hydrolyzate (Oxoid), 3 g; calcium pantothenate, 3.5 g; inositol, 300 mg; cyanocobalamin, 0.05 μg; glycine, 3.0 mg; folic acid, 0.1 μg; nicotinic acid, 1.5 μg; thiamine hydrochloride, 0.1 mg; ferric tartrate, 40 mg; 2,4-dichlorophenoxyacetic acid, 6.0 mg; ascorbic acid, 25 mg, 6-furfurylamino purine (kinetin), 0.1 mg; and naphthalene acetic acid, 1.0 mg; pH 5.9. Subsequent axenic growth was maintained during two subsequent subcultures made at intervals of 20 days on the same fresh medium. Further subcultures produced extremely thin growth. No reinfection studies were reported and before this work can be assessed it will be necessary to see follow-up work published. TUREL (1969) stated that a medium containing 0.1% yeast extract in addition to sucrose, inorganic salts, and chelated iron supported saprophytic growth of *Melampsora lini* (Pers.) Lev., race no. 3. JONES, (1972) obtained axenic culture of carnation rust, *Uromyces dianthi* grown from uredospores on media contained yeast extract, peptone and casein hydrolysate, singly or in combination. He added that spore-like cells were formed in the mycelium of older colonies and these spores were capable of germination and of initiation new saprophytic growth. COFFEY and ALLEN (1973) obtained axenic culture of *Puccinia helianthi* from uredospores on artificial media where this fungus had an obligatory requirement for BSA (bovine serum albumen) and they found also that growth of the flax rust fungus was enhanced in the presence of calcium ions.

It is known that members of the Uredinales have usually been considered the classical examples of obligate parasites amongst fungal plant pathogens. The present work was conducted to investigate to what extent the biotrophic and parasitic fungus *Uromyces fabae,* the causal of broad bean rust, could be able to grow and sporulate under ***in vitro*** conditions. Some attempts for testing pathoginicity of the isolated fungus were also done.

MATERIALS & METHODS

Isolation :

The normal technique for isolating different plant pathogens was used also during this work. Rusted leaves of diseased broad bean plants (*Vicia faba* L.) grown at the Experimental Farm of the Faculty of Agriculture, at Moshtohor, Zagazig Univ., Benha - Branch during 1995 season were collected and surface sterilized by dipping into 2% sodium hypochlorite for approx. 2 min., then removed , thoroughly washed with sterilized distilled water and dried between sterilized filter paper. Single opened and/or closed rust postules were aseptically and carefully removed with little amount of surrounded healthy tissues and placed onto plates containing sterilized PDA medium or MS-medium (MURASHIGE & SKOOG 1962) then incubated for 4-7 days at 20°C. Hyphal tips of the resultant fungal growth(s) on MS-medium were cultured onto PDA plates and incubated as mentioned before. One of the isolated fungi having septate and dicaryotic

mycelium was found to be able to grow on MS- but not on PDA medium. Natural medium contained bee-honey at the rate of 40.0 g/l only or supplemented with pollen grains (collected by honeybees) at the rate of 2.0, 4.0, or 6.0 g/l. were preliminary used for growing the fungal isolate which could not be able to grow on PDA medium. The resultant fungal growth on both media was visually and microscopically examined for mycelium and spore characters to facilitate fungal identification (Grove, 1913 and Singh, 1982).

A very tiny portions of mycelial growth of the latter fungus were transferred from MS-medium onto glass slides with few drops of sterilized tap water and placed into Petri-dishes lined with moistened filter paper and incubated under room conditions for 4-7 days at 20°C., then examined microscopically.

The used modified MS-medium:

In the present work, the medium used was a modification of basal mineral salts of MS-medium described by MURASHIGE and SKOOG (1962). The modified tested basal MS-medium is shown in Table (1).

Table (1): Modification in chemical ingredients of the original MS-medium (Murashige and Skoog's, 1962).

Constituents	Conc. in stock solution (g/L) and in original MS-medioum in mg/L (between bracts)[@]	Final conc. in mg/L. in tested basal medium
NH_4NO_3	82.5 (1650.0)[@]	1650.00
KNO_3	95.0 (1900.0)	13700.00
H_3BO_3	1.24 (6.0)	12.40
K_2PO_4	34.00 (170.0)	340.00
KH_2PO_4	0.166 (0.83)	1.66
$Na_2MoO_4.2H_2O$	0.050 (0.25)	0.50
$CoCl_2.6H_2O$	0.005 (0.025)	0.05
$CaCl_2.2H_2O$	88.00 (440.0)	880.00
$MgSO_4.7H_2O$	74.00 (370.0)	740.00
$MnSO_4.4H_2O$	4.46 (22.3)	44.60
$ZnSO_4.7H_2O$	1.72 (8.6)	17.20
$CuSO_4.5H_2O$	0.005 (0.025)	0.05
Na.EDTA	7.45 (37.35)	74.50
$FeSO_4.7H_2O$	5.57 (27.85)	55.70
Thiamin-HCl	0.02 (0.10)	0.20
Nicotinic acid	0.10 (0.50)	1.00
Pyridoxin-HCl	0.10 (0.50)	1.00
Glycine	0.40 (2.00)	4.00

Effect of dilution of basal medium with additional inositol:

Additional amount 0.2 g/l of inositol was added to the above basal MS-medium. Such full strength medium (N) was diluted with distilled water to prepare 1/2 N, 1/4 N and 1/8 N strengths, respectively. This experiment aimed to know the best strength of medium with or without additional inisitol for *in vitro* growing and sporulation of the iso;ated fungus *Uromyces fabae*.

Effect of adding pollen grains to basal medium:

Each of the above mentioned dilutions of the full strength medium i.e. N, 1/2N, 1/4N and 1/8N with or without additional inositol was supplemented with air dried pollen grains (collected by honeybees) at rate of 10 g/l..

All media were autoclaved at 121°C. for 20 min. and poured after cooling into Petri-dishes of 8.0 cm diameter. The dishes were inoculated each with a 5 mm disk of fungal growth, previously grown for 15 days on the modified MS- medium, then incubated at 20°C. When mycelial growth filled the plates in any particular medium, the fungal linear growth (in mm) as well as sporophore length and uredospores dimensions (in µm) were determined. Average number of uredospers per plate (in a known volume of distilled water) was measured by using the haemocytometer slide. This experiment aimed to know the role of natural amendments on growth and sporulation of *Uromyces fabae* under study. All data were statistically analyzed according to method described by Snedecor and Cochran, (1989).

Effect of MS-medium repeating subcultures on the ability to grow on PDA medium:

Several subcultures of the isolated fungus identified as *Uromyces fabae* were made on the modified MS-medium. Each of these subcultures was resubcultured on PDA medium to find out to what extent this fungus could be adapted to grow saprophytically on PDA.

Pathogenicity test:

Unsporulating mycelial mats grown on modified MS-media were collected separately, blended in sterilized distilled water as well as *Uromyces fabae*-uredospore from sporulating axenic cultures were used for inoculating healthy detached leaves of broad been plants. Inoculated detached leaves, in both tests were incubated under room conditions (approx. 20°C.) and examined daily for 7 days. Healthy broad been plants (30-40 days old) grown under greenhouse conditions were sprayed and/or injected their stems and leaf blade with suspension of the *in vitro Uromyces fabae*-uredospore and daily observed for the development of disease symptoms.

RESULTS & DISCUSSION

Isolation trails:

Isolation trials on MS-medium resulted in different non-sporulating mycelial growth. When these were transferred to PDA medium, *Botrytis sp., Stemphylium sp., Alternaria sp., Tuberculina sp.* could be identified, after an incubation period, according to their mycelial, sporophore and spore characters. An isolated fungal growth with septate mycelium transferred from cultures of MS-medium could never grow on PDA plates. Microscopic examination for the later mycelial growth stained with malachite green showed that some cells of its septate hyphae were dicaryotic i.e. containing two nuclei in addition to some dicaryotic enlarged, rounded cells like-spores. After incubation for 2 weeks at 20°C., the colonies of the later fungal isolate on MS-medium were whitish in color and reached 35-40 mm in diameter.

Formation of postules in In Vitro:

Small parts were taken from the mycelial mats of the aforementioned fungal isolate, placed into few drops of tap water on glass slide and left inside Petri-dishes at 100%R.H. under room conditions for 4-7 days. Microscopic examination

of the latter preparation showed well developed rust postules which could be comparable with rust postules developed naturally on rusted leaves of broad bean.

Effect of beehoney and pollen grains on growth and sporulation of Uromyces fabae:

The obtained results showed that the mycelial growth on agar medium containing beehoney alone was scarcely, pale, very weak and not sporulate. In contrast, mycelial growth on agar medium containing both beehoney and pollen grains was brownish in color, superficially, profusely branched with very dense sporulation. The mycelial growth on the latter medium was obviously increased in addition to formation of few teleutospores like-structures with increasing the added amount of pollen grains up to 6.0 g/l. Mature uredospores produced ***in vitro*** were borne apically on short or long hyaline tapering septate or nonseptate sporophores measuring 5.2 - 8.3 µm in width and 16 - 18 µm in length. Uredospores produced ***in vitro*** were light to brown in color, echinulate or smooth, and measured 17.5 - 22.2 µm (av. 19.85 µm) in width and 17.5 - 26.25 µm (av. 21.88 µm) in length. The ***in vitro*** teleutospores were dark brown, 1-celled and borne apically on hyaline sporophores (Fig.1) as compared with natural cultures. According to the general characters of both uredospores and teleutospores (Fig. 2) and formation of pustules, the isolated fungus was identified as Uromyces fabae (Pers.) De Bary (Singh, 1982).

Effect of additional inositol and pollen grains added to different dilutions of the modified MS-basal medium on Uromyces fabae linear growth and uredospores dimensions:

Data presented in Table (2) and Fig. (3) show that the basal medium containing pollen grains alone or combined with inositol was better for *Uromyces fabae* linear growth than the basal medium alone or combined with inositol only. With regard to strength of medium, data indicated that diluted media at 1/2N, 1/4N or 1/8N was the best for growth of *Uromyces fabae* than the full strength medium i.e. at N concentration.

Table (2): Effect of different amendments and strength of the basal media on *Uromyces fabae* -linear growth (in mm) in axenic culture after incubation for 11 days at 20°C.

	Different strengths of media (concentration)				
Media	N	1/2N	1/4 N	1/8 N	Mean
Basal medium (A)	45.5	51.6	34.6	43.1	43.7
A + Pollen grains	55.0	54.1	56.8	52.4	54.6
A + Inositol	15.5	50.5	44.1	42.1	38.0
A + Pollen grains +Inositol	51.1	52.4	54.6	59.1	54.3
Mean	41.8	52.2	47.5	49.2	

L.S.D. at 5% for:

Media (M) = 2.43
Concentrations (C)= 2.43
M x C = 4.85

However, the highest rate of linear growth of *Uromyces fabae* was obtained by adding both pollen grains and inositol to the basal medium at 1/8N of its full strength. After 11 days of incubation, the lowest *Uromyces fabae* linear growth was observed on full strength basal medium supplemented only with inositol. These results indicated that the used medium may be highly concentrated and its nutritive value to *Uromyces fabae*-growth may be increased by dilution. In this respect, EZEKIEL, (1930) found that host extract was nutritive to *Puccinia graminis* only when very dilute. In fact, inositol may better be placed as metabolites rather than among the vitamins (COCHRANE, 1958 & FRIES, 1965). Accordingly, increasing the amount of inositol added to the used medium seemed to be toxic and inhibited the *in vitro* growth of *Uromyces fabae*.

Table (3): Effect of different amendments and strength of the basal media on number of *Uromyces fabae*-uredospores per plate after incubation for 11 days at 20°C. in axenic culture.

Media	Number of spores/plate at Strength of media (concentration)				Mean
Dilutions	N	1/2 N	1/4 N	1/8 N	
Basal medium (A)	4204	7079	1006	1541	3458
A + pollen grains	13500	11357	8130	4618	9401
A + Inositol	286	6403	1765	3205	2915
A + pollen grains + Inositol	10893	12224	9781	6338	9809
Mean	7221	9266	5171	3926	

L.S.D. at 5% for:

Media (M) = 968.06
Concentrations (C) = 968.06
M x C = 1936.1

Concerning the production of uredospores, data obtained in Table (3) indicate that the addition of inositol alone to most of tested dilutions of the basal

medium caused clear decrease in the number of *Uromyces fabae*-uredospores produced ***in vitro***, while, basal medium with pollen grains only or combined with inositol produced the highest numbers of uredospores. Regardless the used amendments, it was noticed that the number of uredospores on the medium diluted to 1/2N was relatively larger than that produced on media diluted to 1/4N or 1/8N strength. In general, the non diluted (N) basal medium supplied with pollen grains only, 1/2N supplied with both pollen grains and inositol followed by the same dilution supplied with pollen grains only were promising for formation of uredospores in axenic culture. In contrast, the lowest number of uredospores was produced on the non diluted (N) basal medium supplemented with inositol only. Promising fungal growth and sporulation on basal medium supplemented with pollen grains may be attributed to hormonal effects of the pollen grains which prevent damage due to strength of the used concentrated medium or inhibited the toxic effects of inositol.

Table (4): Effect of different media and media strength on average** length of Uromyces fabae- uredospores (in µm) in axenic culture.

Tested amendments	Strengths (concentrations) of media				
	N	1/2 N	1/4 N	1/8 N	Mean
Basal medium (A)	24.76	23.36	23.52	24.59	24.06
A + pollen grain	22.80	22.73	23.85	24.14	23.38
A + Inositol	23.79	23.53	24.48	23.23	23.76
A+ pollen grains + Inositol	23.65	23.52	23.45	21.92	23.14
Mean	23.75	23.29	23.83	23.47	
Control (Natural spores)	23.94				

(** The average of 100 measurements replicated four times)

L.S.D. at 5% for:

Media = 0.536
Concentrations = 0.599
M x C = 1.198

Table (5): Effect of different media and media strength on average** width of *Uromyces fabae*- uredospores (in µm) in axenic culture.

Tested amendments	Strengths (concentrations) of media				
	N	1/2 N	1/4 N	1/8 N	Mean
Basal medium (A)	22.49	21.22	21.89	22.80	22.10
A + pollen grain	21.40	20.72	21.95	22.28	21.59
A + Inositol	21.83	21.92	22.02	21.4	21.79
A + pollen grains + Inositol	21.39	21.71	21.20	20.40	21.18
Mean	21.78	21.39	21.77	21.72	
Control (Natural spores)	23.75				

(** The average of 100 measurements replicated four times)

L.S.D. at 5% for:

Media (M) = 0.346
Concentrations (C) = 0.387
M x C = 0.773

Table (6): Effect of different media and media strength on average** wall thickness of *Uromyces fabae*- uredospores (in µm) in axenic culture.

Tested amendments	Strengths (concentrations) of media				
	N	1/2 N	1/4 N	1/8 N	Mean
Basal medium (A)	2.38	2.30	2.48	2.45	2.40
A + pollen grain	2.38	2.35	2.63	2.20	2.39
A + Inositol	2.30	2.48	2.15	2.63	2.39
A+ pollen grains + Inositol	2.05	2.18	2.20	2.63	2.27
Mean	2.28	2.33	2.37	2.48	
Control (Natural spores)	1.75				

(** The average of 100 measurements replicated four times)

L.S.D. at 5% for:

Media (M) = 0.136
Concentrations (C) = 0.152
M x C = 0.303

Regarding the dimensions of *Uromyces fabae*-uredospores data presented in Tables (4 and 5) show that the length and width of *Uromyces fabae* uredospore ranged between 21.92 - 24.76 µm and 20.40 - 22.80 µm, respectively. The highest length and width of uredospores were produced on the non diluted (N) and 1/8N diluted basal medium, respectively, while the lowest values for both measurements were associated with uredospores formed on 1/8N diluted basal medium provided with both pollen grains and inositol. The data in Table (6) showed that wall thickness of uredospores ranged between 2.05 - 2.63 µm**.** Wall thickness seems to be slightly or not affected neither by dilutions of the tested media nor by inositol and/or pollen grains added to them. However the highest wall thickness was associated with dilution's of 1/4N and 1/8N provided with both pollen grains and inositol compared with the normal non diluted medium (N) provided with the same amendments. These findings are in agreement with SINGH (1982) who indicated that the natural uredospores of *Uromyces fabae* are round to ovate, light-brown, echinulate and measured 20-30 (22-28) x 18-26 (19-22) µm.

Pathogenicity test:

Typical rust disease symptomes were never developed either on intact broad bean plants under greenhouse conditions or on their detached leaves under *in vitro* conditions either sprayed with blended mycelial mats of *Uromyces fabae* or its uredospores taken from fungal growth on MS-medium. Microscopic examination for epidermal strips of the inoculated leaves showed minuet areas containing hyaline cells-like spores. The majority of uredospores were found not be able to germinate on surfaces of inoculated broad bean leaves. Such observed lackadaisical pathogenicity could be attributed to several factors such as separation of avirulent strain during isolation processes, adaptation to grow saprophytically, induced mutation, sensitivity of uredospores to phytoalexins produced by inoculated leaves or inhibition effects of micoflora associated with broad bean leaves. In this regard, STAKMANN & CHRISTENSEN (1953) indicated that mutation rates as high as 0.8 per cent for parasitic fungi, some of these mutations can be expected to affect the culturability of these species. It is probable that different species within each of the taxonomic groups are considered to comprise obligate parasites will show

different responses to nutritional treatments. Williams et al., (1967) stated also, that successful reinfection by spores formed in axenic culture was obtained only when uredospores of *Puccinia graminis* f.sp. *tritici* were placed under the epidermis in contact with mesophyll cells. When placed directly on the intact leaf, spores gave a high percent germination but germ tube elongation was limited. Occasionally, appressoria formed over stomata but did not develop further. It seems that the inability of uredospores to infect hosts by stomatal invasion is due to a low potential for independent growth rather than a lack of pathogenicity. Parker and Blakeman (1984) found also, that growth of germ-tubes of *U. viciae-fabae* urediniospores was appreciably reduced on previously-wetted broad bean leaves compared with previously dry leaves.

Finally, the present success towards growing and sporulating *U. fabae* in axenic culture might be very important especially in programs of plant breeding for disease resistance rather than understanding its physiological behavior and nutritional requirements under in vitro conditions. This accomplishment, however, needs further studies and necessitates new appraisal of the culturability of of this obligatory pathogen.

Fig.(1): Formation of uredospores and teleutospores on agar medium containing beehoney and pollen grains (400 X). 1- Uredospores 2- Teleutospore

Fig. (2) Comparative morphology between natural uredospores (1) and teleutospores (3) from naturally infected broad bean plants and uredospores (2) and teleutospores (4) growing in axenic culture. (600 X).

N 1/2N 1/4N 1/8N

A

A+P

A+I

A+P+I

Fig.(3):Showing effect of different amendments and strength of the basal media on *Uromyces fabae* -linear growth (in mm) in axenic culture after incubation for 11 days at 20°C. (A = Basal medium , A+P = A + Pollen grains, A+I = A + Inositol, and A+P+I = A + Pollen grains +Inositol).

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