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Colonization of *Retama raetam* seeds by fungi and their significance in seed germination

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Examination by scanning electron microscopy and incubation on potato-dextrose agar medium showed that dry seeds of *Retama raetam* were externally free of fungi. When planted in sandy loam soil, the seeds become colonized with eleven soil-borne fungal species. The fungi were isolated on cellulose agar, pectin agar and lignin agar media. *Aspergillus flavus, A. niger, A. fumigatus, Penicillium capsolatum* and *Fusarium oxysporum* had broad occurrence and were recovered on all the three media. The production of hydrolytic enzymes by the isolated fungi depends on the substrate and species. *Penicillium capsolatum, P. spinulosum* and *A. niger* had wide enzymatic amplitude and they were able to produce cellulolytic, pectolytic and lignolytic activities on corresponding substrates as well as on seed-coat-containing media. The lignolytic activities of the isolated species except *Chaetomium bostrychodes* and *Trichoderma viride* were enhanced by applying the seed-coat materials as C- source rather than lignin. Soaking *R. raetam* seeds in culture filtrates of most of the fungi grown on seed-coat-supplemented media induced a pronounced and distinct stimulating effect on seed germination. The most effective filtrates were those of *P. capsolatum, P. spinulosum* and *Sporotrichum pulverulentum*.

Key Words——hydrolytic enzymes; mycoflora; *Retama raetam*; seed-borne fungi.

Introduction

Retama raetam Forssk Webb (Leguminosae) is a medicinal shrub of economic importance growing under adverse arid conditions in the western Mediterranean belt, northern Arabian desert and Sinai. *Retama raetam* has been found to produce a tremendous number of seeds, reaching 12037 per plant (Zayed, 1983). Despite the production of this large number of seeds, it is not frequent to observe *R. raetam* seedlings in the desert. It seems that the germination of these seeds is very limited, ranging from 2 to 3% (Zayed, 1983). The main reason for such a low germination is the hard seed coat. As an artificial means, sulphuric acid scarification (Vora, 1989) and boiling temperature (Washitani, 1988) have been conventionally employed to soften hard seeds and increase seedling emergence. However, living cells of higher plants cannot normally withstand high temperature outside a certain physiological range (Steponkus, 1982). As an alternative, biological treatment may offer a more practical approach to the improvement of germination of hard-coated seeds.

Seed germination in the soil is accompanied by a period of intense microbial activity, both on the seed coat surface and in the soil immediately surrounding the seed (Verona, 1963). The volatile compounds evolved from germinating seeds may support the growth of a variety of bacteria and fungi, and this is due to the nutritive value of the volatiles (Schenck and Stotzky, 1975). The developing microorganisms may accelerate the seed germination, probably by hastening breakdown of the seed coat (Guttridge et al., 1984). Several investigations in recent years have revealed the possible involvement of hydrolytic enzymes produced by fungi in biodegradation of plant materials.

The objective of this study was to determine the fungal colonization of soil-germinated *R. raetam* seeds. The in vitro ability of the isolated species to produce hydrolytic enzymes and the effect of the enzyme-containing filtrates on breaking the dormancy of the seeds were also studied.

Materials and Methods

Seed Samples Pods were collected in 1991 from *Retama raetam* plants grown in wadis (5 localities) of northern Sinai, Egypt. In the laboratory the seeds were removed under aseptic conditions from the pods and stored at 26°C until use.

Scanning electron microscope (SEM) The SEM was used to detect the presence of fungal spores or mycelia on the seed. The seed coat of *R. raetam* was broken in a mortar and pestle and separated by winnowing. The seed coat was then freeze-dried, gold coated and observed with a Jeol 35C scanning electron microscope. Twenty seeds were used.

Isolation of fungi This experiment was conducted to determine the different types of fungi colonizing the seed coat during seed germination in the soil. About 150 seeds were sown in each of a series of 18 25-cm pots

containing sandy loam soil. The soil was collected from the garden of the Botany Department, Cairo University. The high number of seeds in each pot is due to the low germination rate of about 2–3%. The experiment was run in triplicate. After 10 d the germinated seeds were removed under aseptic condition, washed with sterile water to remove the adherant soil particles and the seed coat was carefully separated. The seed coats were transferred to a series of 9-cm Petri plates, each containing about 15 ml of medium containing cellulose, pectin or lignin (3 seed coats/plate). Three sets of 6 plates each were used for each type of medium. The plates were incubated at 26°C and the developing fungi were identified using the publications of Gilman (1957), Barron (1968) and Barnett and Hunter (1972).

Chemical analyses of seed coat The seed coats were separated by the method described in SEM. The oven-dried seed coats were ground in a UDY (Ft. Collins, Co, USA) cyclone mill using a 1-mm sieve. Neutral detergent fiber, acid detergent fiber (ADF) and lignin were determined in duplicate using sodium lauryl sulfate, cetyl trimethylammonium bromide and 72% sulfuric acid solutions (Goering and Van Soest, 1970). Cellulose values were determined as the difference between ADF and lignin values. Pectin was extracted with hydrochloric acid (Huang, 1973), assayed colorimetrically (Blumenkranz and Asboe-Hasen, 1973), and expressed as the amount of galactouronic acid per 1 g dry seed sample.

Hydrolytic enzymes Lignolytic activity was determined according to Betts et al. (1987). The medium contained 0.5 g of $NaNO_3$, 0.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of yeast extract, and 1.0 g of lignin substrate (lignin or seed coat material) in 1000 ml of distilled water. The lignin substances were dissolved separately and mixed with the medium prior to adjusting the pH to 5.0. The medium was dispensed into 250-ml conical flasks, each containing 50 ml. The flasks were inoculated with a 5-mm mycelial disc of 5-d-old culture. Five replicate flasks for each lignin substance were used for each fungus. Duplicate flasks without inoculum served as controls. The flasks were incubated at 28°C for 8 d. The enzyme activity in the media was determined in terms of the cleavage of lignin in the reaction mixture at 30°C. It was defined as the increase in total phenol (μg) in 1 ml reaction mixture/h. The total phenols were measured using the colorimetric method of Folin-Denis as described by Swain and Hillis (1959).

For determination of pectolytic activity, the growth medium contained 2.5 g of KH_2PO_4, 10 g of NaCl, 10 g of pectic substance (pectin or seed coat materials) in 1000 ml of distilled water. The pectinase activity in the media was determined by the viscometric method described by Talboys and Busch (1970) using an Ostwald viscometer. The values were expressed as percentage reduction in viscosity of 1% pectin solution.

Mineral salt-carboxymethylcellulose (CMC) was used for induction of cellulase. The cellulolytic activity in the culture filtrates was determined by the viscometric method (Abdel-Razik, 1970). It was expressed as percentage reduction in viscosity of 1% CMC.

Seed germination A portion of the enzyme containing filtrates of isolated fungi was transferred to a sterile bottle. About 100 seeds of *R. raetam* were soaked for 12 h in each bottle. Five bottles were used for each fungal filtrate. At the end of the soaking period the seeds were removed, washed with sterile water and transferred to moist filter paper in sterile plates. Care was taken to keep the filter paper moist. The plates were incubated at 26°C. A seed was considered to have germinated when the radicle emerged. The seed germination was calculated as percentage of total seed used.

The data shown in Tables 1 and 2 were analysed statistically in terms of the standard deviation, and those in Tables 3 and 4 in terms of least significant difference (LSD).

Results and Discussion

Isolated fungi Examination by scanning electron microscopy showed that the dry unplanted seeds of *R. raetam* were externally free of fungi. This finding was supported by cultivation of the seeds on potato-dextrose agar medium. It seems that the seed-borne mycoflora depends on the nature of the seed coat. Thick, hard, smooth coats with low moisture-holding capacity were associated with fewer fungi (Nair, 1982). When planted in sandy loam soil, the germinated seeds became colonized by eleven soil-borne fungal species (Table 1). *Aspergillus flavus* Link: Fr., *A. niger* Tiegh., *A. fumigatus* Fresen., *Penicillium capsolatum* Raper & Fennell and *Fusarium oxysporum* Schlehtend.: Fr. occurred widely and were recovered on the three types of media used. They were more frequent in cellulose than lignin or pectin media. The first two species were more common than the latter ones. The recovery of these species from the seed coat on the different media indicates that they have wide nutritional and enzymatic amplitude and could utilize the different components of seed coats and/or leachate. These fungi might not be involved in the initial breakage of the seed coat but may hasten its rupture after softening of the coat by hydrolytic enzymes of the seed. The solubilization of the insoluble materials of the seed coat by the seed enzymes is an important mechanism of seed softening and is usually associated with colonization by fungi which utilize the released soluble components of the seed and might take part in decreasing seed coat firmness. Lisker et al. (1985) showed that in cracked seed coats and in damaged areas on broken soybeans, profusely developing fungal mycelia were frequently observed although the intact seeds were externally free of microorganisms. They also noticed that pentrating fungi were responsible for the increase of free fatty acids in the seed.

Enzyme activities With the exception of *Chaetomium bostrychodes* Zopf and *Trichoderma viride* Pers.: Fr., the seed coat of *R. raetam* enhanced the in vitro lignolytic activity of the other isolated fungi in comparison with media supplemented with lignin (Table 3). This indicates the specificity of these fungi to degrade seed-coat lignin, and it is reasonable, therefore, for them to be isolated from

Table 1. Count (Co, colony per seed) and frequancy of occurrence (FO, out of 18) of fungal species recovered on germinated seeds of *Retama raetam*.

Fungal species	Agar medium supplemented with					
	Cellulose		Pectin		Lignin	
	Co	FO	Co	FO	Co	FO
Chaetomium bostrychodes	0.1±0.02	10	0.0	0	0.0	0
Humicola fuscoatra	2.1±0.62	11	0.0	0	0.0	0
Aspergillus flavus	3.1±0.75	13	1.1±0.42	5	2.3±0.69	10
A. niger	2.9±0.73	10	1.4±0.43	5	1.7±0.70	9
A. fumigatus	2.0±0.63	8	0.9±0.08	3	1.6±0.70	6
Trichoderma viride	0.9±0.07	11	0.0	0	0.0	0
Fusarium oxysporum	2.6±0.70	6	1.6±0.43	4	1.4±0.71	5
Paecilomyces varioti	0.7±0.05	2	0.0	0	1.2±0.70	8
Penicillium capsolatum	2.1±0.66	7	2.0±0.47	8	1.2±0.71	6
P. spinulosum	0.7±0.05	3	0.0	0	1.0±0.05	8
Sporotrichum pulverulentum	0.4±0.04	3	0.0	0	0.7±0.04	7
Total count	18.7		7.0		12.5	

±: Standard deviation.

Table 2. Chemical composition (mg/g) of some components in the coat of *Retama raetam* seed.

Material	Content
Neutral detergent fiber	431±23.3
Acid detergent fiber	405±28.6
Cellulose	299±19.2
Pectin	42±06.8
Lignin	106±13.7

±: Standard deviation.

the seed coat, which was found to contain a considerable amount of lignin (Table 2). *Sporotrichum pulverulentum* followed by *P. capsolatum* and *P. spinulosum* Thom were the Novobranova best ligninase producers. The relatively low count of *S. pulverulentum* on lignin containing medium, in spite of its high lignolytic activity may be due to reduction in its growth and sporulation on the seed coat. The correlation between fungal growth and enzyme activity was studied by Fahmy and Ouf (1993). They found that the pectolytic activities of *Penicillium funiculosum* Thom and *P. oxalicum* Currie & Thom were enhanced although there was a significant reduction in their growth.

The pectolytic activity of *A. flavus* was nullified on the seed coat-supplemented medium, while it is promoted on the same medium in *A. fumigatus* and *F. oxysporum*. The cellulolytic activity of *C. bostrychodes* was appreciably reduced on changing the substrate from cellulose to seed-coat material. However this change favoured enzyme induction by *P. capsolatum*. The results indicate that the production of hydrolytic enzymes depends on the substrate and fungal species. Petruccioli and Servili (1987) found that *Cryptococcus albidus* (K. Saito) C.E. Skinner var. *albidus* exhibited a characteristic ability to grow and produce high level of pectolytic activity on liquid medium containing meal made from sunflower calathides as the sole carbon source.

Penicillium capsolatum, P. spinulosum and *A. niger* had wide enzymatic amplitude. They were able to grow and produce hydrolytic enzymes on different substrates. The former species was the most inductive one. Although *Chaetomium* is a well-known cellulolytic enzyme producer whose cellulolysis of such natural products as bagasse and wheat straw is higher than that of pure cellulose (Lakshmikant, 1990), its cellulolytic activity clearly dropped when the seed-coat material was used as substrate. This drop may attributed to the inaccessibility of cellulose due to the presence of lignin. This observation substantiates the data formerly reported by Bowen and Harper (1990). They found that *Chaetomium globosum* Kunze: Fr. could degrade cellulose but not lignin, also that it can demethoxylate lignin and thus gain access to protected polysaccharides. Also, it is noted that some fungi such as *S. pulverulentum* and *F. oxysporum* were isolated from the seed coat of *R. raetam* seeds on cellulose agar medium although they are not cellulase producers. These fungi may depend on the decomposed

Table 3. Hydrolytic enzyme activity of fungi isolated from the coats of *Retama raetam* seeds, when cultured on media supplemented with the corresponding substrate or seed-coat materials.

Fungal species	Cellulolytic activity (loss in viscosity) on media supplemented with		Pectolytic activity (loss in viscosity) on media supplemented with		Lignolytic activity (μg phenol/ml reaction mixture/h) on media supplemented with	
	cellulose	seed coat	pectin	seed coat	lignin	seed coat
Chaetomium bostrychodes	89.3	22.1	0.0	0.0	0.0	0.0
Humicola fuscoatra	85.2	28.3	0.0	0.0	106.0	139.6
Aspergillus flavus	38.1	45.2	14.6	0.0	93.6	118.5
A. niger	6.8	5.1	11.2	9.6	38.2	109.2
A. fumigatus	0.0	0.0	9.1	21.8	76.2	121.2
Trichoderma viride	36.7	28.7	5.8	9.3	31.2	0.0
Fusarium oxysporum	0.0	0.0	18.9	6.3	182.1	280.7
Paecilomyces varioti	0.0	0.0	0.0	0.0	106.2	149.1
Penicillium capsolatum	41.6	66.3	23.1	27.9	112.6	301.2
P. spinulosum	25.0	32.3	23.9	27.0	96.1	196.2
Sporotrichum pulverulentum	0.0	0.0	0.0	0.0	298.5	266.0
Least significant difference at 5%	6.8	4.2	4.9	3.8	12.8	22.9

Table 4. Effect of seed soaking in filtrates of fungi, grown on different substrates, on percentage seed germination of *Retama raetam*.

Fungal species	Percentage seed germination			
	Filtrates recovered from media supplemented with			
	seed coat	cellulose	pectin	lignin
Chaetomium bostrychodes	3.8	3.8	3.8	3.8
Humicola fuscoatra	10.6	5.6	3.8	7.8
Aspergillus flavus	14.6	6.2	4.6	6.4
A. niger	10.8	3.8	3.8	5.0
A. fumigatus	13.8	3.8	3.8	5.0
Trichoderma viride	4.0	4.8	3.8	3.8
Fusarium oxysporum	16.2	3.8	3.8	8.8
Paecilomyces varioti	8.6	3.8	3.8	8.0
Penicillium capsolatum	28.6	6.2	4.2	8.0
P. spinulosum	25.0	6.0	5.2	8.2
Sporotrichum pulverulentum	20.0	3.6	3.8	11.8
Least significant difference at 5%	4.1	3.8	2.9	3.5

materials formed by other species.

Seed soaking The culture filtrates of the fungi grown on seed-coat materials, except in the case of *C. bostrychodes* and *T. viride*, have a pronounced and distinct stimulating effect on seed germination of *R. raetam* (Table 4). The most effective filtrates were those of *P. capsolatum, P. spinulosum* and *S. pulverulentum*. This is attributable, at least in part, to the high lignolytic activity contained in the filtrates of these fungi. The former two species also contain pectolytic and cellulolytic enzymatic activities, which increase the digestibility of seed coats and improve the seed germination. It was found that soaking the seeds of jowar, maize, wheat and paddy in mixed culture filtrates of some fungi and *Azotobacter* increased germination by 2-13% as compared to culture filtrates of pure *Azotobacter* (Mallikar-junaiah and Bhide, 1985). The culture filtrates of fungi grown on media supplemented with pectin or cellulose were inadequate to support a significant rise in percentage seed germination. However, the effect of fungal filtrates of lignin-supplemented media on seed germination is correlated with the lignolytic activity: whenever this activity increases, the percentage seed germination increases.

The culture filtrates of fungi grown on seed coat-containing media are more suitable for seed soaking to enhance germinability than those of lignin media. Guttridge et al. (1984) showed that the germination of strawberry seeds was variously promoted by natural and artificial infection of *Ulocladium chartarum* (G. Preuss) E. Simmons and *Cladosporium* sp. They found that the germi-

nated seedlings were apparently undamaged by these infections although when cultured on malt agar the seedlings were overgrown by the fungi.

It is concluded that the low germinability of *R. raetam* seeds may be due, at least in part, to the reduced mycoflora of its natural sandy habitat and the absence of fungi producing hydrolytic enzymes that may have a role in hastening germination. The results obtained point to the advantage of cultivation of seeds in fungal-rich sandy loam soil rather than fungal-poor sandy soil. Also, the amendment of the soil with lignin-decomposing fungi may result in improvement of the seed germination of the target plant.

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