THE KOREAN JOURNAL OF MYCOLOGY
Vol. 21, No 4, p316-322 December 1993

Printed in S. KOREA

Colonization of *Retama raetam* Seeds by Fungi and Their Significance in Seed Germination

OUF S.A.*

Botany Department, Faculty of Science, Gairo University, Giza, Egypt.

ABSTRACT: Examination by scanning electron microscopy and potato-dextrose agar medium showed that the dry seeds of *R. raetam* were externally free of fungi. When planted in sandy loam soil, the seeds become colonized with eleven soilborne fungal species. The fungi were isolated on cellulose agar, pectin agar and lignin agar media. *Aspergillus flavus, A. niger, Penicillium capsulatum* and *Fusarium oxysporum* had broad occurrence and recovered on the three media. The production of hydrolytic enzymes by the isolated fungi depends on the substrate and species. *P. capsulatum, 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 bostrychods* and *Trichoderma viride* were enhanced on applying the seed coat materials as C-source rather than using lignin. Soaking *R. raetam* seeds in culture filtrates of the most fungi grown on seed coat supplemented media induced pronounced and distinct stimulating effect on seed germination. The most effective filtrates were those of *P. capsulatum, P. spinulosum* and *Sporotrichum pulverulentum*.

KEYWORDS: ***Retama raetam* seed, seed coat, germination, fungi.**

Retama raetam Forssk Webb is a medicinal shrub of economic importance growing under adverse arid condition in the western mediterranean belt, northern arabian desert and Sinai. *R. raetam* has been found to produce 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 seedlings emergence. Living cells of higher plants, however, cannot normally withstand high temperature outside certain physiological range (Steponkus, 1982). As an alternative, biological treatment may offer a more practical approach toward the improvement of germination of hard coated seeds.

Seeds 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 was also studied.

*Corresponding author

Materials and Methods

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

Scanning electron microscope(SEM) : It 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 35℃ scanning electron microscope. Twenty seeds were used.

Isolation of fungi : This experiment was conducted to determine the different types of fungi colonized on the seed coat during seed germination in the soil. The seeds were sown in a series of 18, 25 cm pots containing sandy loam soil, about 150 seed/pot. The soil was collected from the garden of Botany Department, Cairo University. The high number of seeds in each pot is due to the low percentage germination which range between 2−3%. The experiment was run in triplicate. After 10 days the germinated seeds were removed under aseptic condition, washed with sterile water to remove the adherant soil particles and the seed coat was carfully separated. The seed coats were transferred to a series of 9 cm Petri plates, each contains about 15 m*l* cellulose, pectin and lignin containing media(3 seed coats/plate). Three sets, each of 6 plates were used for each type of medium. The plates were incubated at 26℃ and the developing fungi were identified using the publication 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 was determined by difference using ADF and lignin values. The extraction of pectin was accomplished by hydrochloric acid (Huang 1973). Its amount was assessed colorimetrically (Blumenkranz and Asboe-Hasen, 1973) and calculated 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 $NaNO_3$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ 1.0 g yeast extract, 1.0 g lignin substrate (lignin or seed coat material) in 1000 m*l* distilled water. The lignin substances were dissolved separately and then mixed with the media prior to adjusting the pH to 5.0. The medium was dispensed into 250 m*l* conical flasks, each contained 50 m*l*. The flasks were inoculated with a 5 mm mycelial disc of 5 day old culture. Five replicate flasks for each lignin substance were used for each fungus. Other duplicate flasks without inoculum were served as control. The flasks were incubated at 28℃ for 8 days. The enzyme activity determined in the media and depends on the cleavage of lignin in the reaction mixture at 30℃. It was defined as the increase in total phenol(μg) in 1 m*l* 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 KH_2PO_4, 10 g NaCl, 10 g pectic substance (pentin or seed coat materials) in 1000 m*l* distilled water. The pectinase activity was determined in the media by using the viscometric method as described by Talboys and Busch(1970). The method depends on the reduction in viscisity of 1% pectin solution in Ostwald's viscometer. The values were expressed as percentage reduction in viscosity of pectin.

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 loss 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.*

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 capsulatum	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 dry matter) of some components in the coat of *Retama raetam* seed.

Material	Chemical composition
Neutral detergent fiber	431± 23.3
Acid detergent fiber	405± 28.6
Cellulose	299± 19.2
Pectin	42± 6.8
Lignin	106± 13.7

± Standard deviation

raetam were soaked for 12 h in each bottle. Five bottles were used for each fungal filtrate. By 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 maintain the filter paper moist. The plates were incubated at 26℃. 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 by using the standard deviation while those in Tables 3 and 4 by using 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. The freedom of the seeds from fungi was supported by cultivation of the seeds on potato-dextrose agar medium. It seems that the seedborne mycoflora depends on the nature of seed coat. Thick, hard, smooth ones and low moist holding capacity were associated with fewer fungi(Nair, 1982). When planted in sandy loam soil, the germinated seeds become colonized with eleven soilborne fungal species(Table 1). *Aspergillus flavus, Humicola fuscoatra, A. niger, Penicillium capsulatum* and *Fusarium oxysporum* have broad occurrence and recovered on the three types of media used. They are more frequent in cellulose than lignin or pectin media. The first three species were more common than the later two. The recovery of these species from the seed coat on different media

Table 3. Hydrolytic enzyme activity of the fungi isolated from the coat 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/1 m*l* 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 capsulatum	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 capsulatum	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

used indicates that they have wide nutritional and enzymatic amplitude and they could utilize the different components of seed coats and/or leachate. These fungi might not be involved in the intial breakage of seed coat but may hasten its rupture after softeness of the coat by hydrolytic

enzymes of the seed. The conversion of the insoluble materials of the seed coat to soluble ones, by the seed enzymes, is an important mechanism of seed softenining and this is usually associated with fungal colonization 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 penetrating fungi were responsible for the increase of the free fatty acids in the seed.

Enzyme activities : With exception of *C. bostrychodes* and *Trichoderma viride*, the seed coat *R. raetam* enhanced the *in vitro* lignolytic activity of the other isolated fungi on comparison with supplemented with lignin (Table 3). This indicates the specificity of these fungi to degrade the seed coat lignin and so it is reasonable to be isolated from the seed coat which was found to contain considerable amount of lignin(Table 2). *S. pulverulentum* followed by *P. capsulatum* and *P. spinulosum* were the most ligninase producers. The relative 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* and *P. oxalicum* were enhanced although there was a significant reduction in their growth.

The pectolytic activity of *A. flavus* was nulified on the seed coat supplemented medium, while it is promoted on the same medium by *A. fumigatus* and *F. oxysporum*. The cellulolytic enzyme of *C. bostrychodes* was appreciably checked on changing the nature of substrate from cellulose to seed coat material. However, this change favoured enzyme induction by *P. capsulatum*. It appears from the results that the production of hydrolytic enzymes depends on the substrate and fungal species. Petruccioli and Servili(1987) found that *Cryptococcus albidus 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 sole carbon source.

P. capsulatum, 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 well known cellulolytic enzyme producer and its cellulolysis increases towards the natural products as bagasse and wheat straw more than pure cellulose(Lakshmikant 1990), but there were a clear drop in its cellulolytic activity on using the seed coat material as substrate. This drop may be attributed to the inaccessibility of cellulose due to the presence of lignin. This observation sustantiates the data formely reported by Bowen and Harper(1990). They found that *Chaetomium globosum* could degrade cellulose but not lignin, also it can demethoxylate lignin and thus gain access to protect 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 ready decomposed materials formed by other species.

Seed soaking : The culture filtrates of the fungi grown on seed coat materials, except *C. bostrychodes* and *T. viride* have pronounced and distinct stimulating effect on seed germination of *R. raetam*(Table 4). The effective filtrates were those of *P. capsulatum, P. spinulosum* and *S. pulverulentum.* This is attributable at least to the high lignolytic activity contained in the filtrates of these fungi. Moreover, the former two species contain pectolytic and cellulolytic enzymatic activities which increase the seed coat degestibility 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 increase germination by 2−13% as compared to culture filtrates of pure Azotobacter(Mallikarjunaiah and Bhide, 1985). The culture filtrates of fungi grown on pectin or cellulose supplement media were inadequate to support significant rise in the percentage of seed germination. However,

the effect of fungal filtrate of lignin amended media on seed germination is correlated with the lignolytic activity, whenever it is more, the percentage of seed germination increases.

The culture filtrates of fungi grown on seed coat contained media is more suitable for used soaking and germinability than those grown on lignin ones. Guttridge *et al*(1984) showed that the germination of strawberry seeds was variouly promoted by natural and artificial infection of *Ulocladium chartarum* and *Cladosporium* sp.. They found that the germinated seedlings were apparentaly undamaged by thses infection although the seedlings, cultured on malt agar, 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 hydrolytic enzymes producing fungi which may have a role in hastening the seed germination. The results obtained encourage the cultivation of the seeds in fungal-rich sandy loam soil rather than fungal poor sandy soil. As well the amendment of the soil with some lignin-decomposing fungi may result in improvement of the seed germination of the target plant.

Acknowlegment

The author is grateful to Dr. G. M. Fahmy(Botany Department, Faculty of Science, Gairo University) for help in collection of samples and suggestions.

References

Abdel-razik, A. A. 1970. The parasitism of *Sclerotium cepivorum* Berk. Ph. D. Thesis., Fac. Agric., Assuit Univ. Egypt.

Barnett, H. L., Hunter, B. B. 1972. Illustrated Genera of Imperfect Fungi. Burgess publishing Company, Minneopolis.

Barron, G. L. 1968. The Genera of Hyphomycetes from Soil. The Williams & Wilkings Company, Baltimore.

Betts, W. B., Ball, M. C., Dart, R. K. 1987. Growth of *Aspergillus flavus* on an insoluble lignin model. *Trans. brit.* mycol. Soc. **89**: 235.

Blumenkranz, N., Asboe-hasen, B. 1973. New method for quantitative determination of uronic acid. *Anal Biochem.* **54**: 484-489.

Bowen, R. M., Harper, S. H. T. 1990. Decomposition of wheat straw and related compounds by fungi isolated from straw in arable soil. *Soil Biol. Biochem.* **22**: 393-399.

Fahmy, G. M., Ouf, S. A. 1992. The mucilage gland of *Limonium delicatulum*(Plumbaginaceae): Secretion and fungal colonization. *Zagazig J. agr. Res.*: **19**: 789-804.

Gilman, J. C.: A Manual of Soil Fungi. 1957. Iowa State Univ. Press, Ames.

Goering, H. K., VAN Soest, P. J. 1970. Forage fibre analysis(apparatus, reagents, procedures and some applications). USDA Handb. No. 379, ARS, USDA, Washington, D. C.

Guttridge, C. G., Woodly, S. E., Hunter, T. 1984. Accelerating strawberry seed germination by fungal infection. *Ann. Bot.* **54**: 223-230.

Huang, J. M. 1973. Improved method for the extraction of pectin. *Proc. Florida State Hort. Soc.* **86**: 260-261.

Lakshmikant 1990. Cellulose degradation and cellulose activity of five cellulolytic fungi. *World J. Microbiol. Biochem* **6**: 64-66.

Lisker, N., Ben-efratim, A., Henis, Y. 1985 Fungi growing on stored soybeans and their significance in lipid breakdown. *Ann. Appl. Biol.* **107**: 117-126.

Mallikar-junaiah, R. R., Bhide, V. P.: Effect of fungi on seed germination in association with *Azotobacter.* Zentralblatt für Mikrobiologie, **140**: 3, 205-211(1985).

Nair, L. N.: Studies on mycoflora of seeds 1982. Some Cucurbitaceous vegetables. *J. Indian Bot. Soc.* **61**: 342-345.

Petruggioli, M. Servili, M. 1987. Pectic enzyme production by *Cyptococcus albidus var. albidus* growing on sunflower calathide meal. Annali della Facolta di Agraria, Universita degli Studi di Perugia **41**: 9-15.

Schengk, S. Stotzky, G. 1975. Effect on microorganisms of volatile compounds released from germinating seeds. *Can. J. Microbiol.* **21**: 1622-1634.

Steponkus, P. L. 1982. Responses to extreme temperatures. Cellular and sub-cellular bases. In Encyclopedia of Plant Physiology 12A Physiological Plant

Ecology, pp. 372-401. eds Lang, O., Osmond, C. B., Nobel, P. S. Berlin: Springer.

Swain, T., Hillis, W. E. 1959. The quantitative analysis of phenolic constituent. *J. Sci. Food Agric.* **10**: 63.

Talboys, P. W., Busch, L. V. 1970. Pectic enzyme produced by *Verticellium* species. *Trans brit. mycol. Soc.,* **55**: 367-381.

Berona, O.: Interaction entre la graine en germination et les microorganismes telluriques. *Ann. Inst. Pasteur.* Paris. **105**: 75-98.

Vora, R. S. 1989. Seed germination characteristics of selected native plants of the lower Grande Velley, Texas Journal of Range Management **42**: 36-40.

Washitani, I. 1988. Effect of high temperatures on the permeability and germinability of the hard seeds of *Rhus javanica* L., *Ann. Bot.* **62**: 13-16.

Zayed, M. A. 1983. Ecological study on Retama raetam in the Egyptian desert. M. Sc. Thesis, Fac. Sci., Cairo Univ., Cairo.