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Research Article

Mycoflora in Exhumed Seeds of *Opuntia tomentosa* and Its Possible Role in Seed Germination

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The funicular cover of the *Opuntia tomentosa* seed limits imbibition; germination occurs only when the funicle is weakened or the funicular valve is removed. We investigated the role of fungi in funicular weakening and seed germination. Seeds that had been either buried in one of two sites or stored in the laboratory were germinated with and without a valve. Disinfected or nondisinfected seeds and their naked embryos were cultivated on agar or PDA. None of the 11 identified fungal genera grew on the disinfected control seeds or the embryos. The mycoflora present on disinfected and nondisinfected exhumed seeds suggest that the fungal colonization occurred in the soil and differed between the burial sites. Exhumed seeds with and without a valve germinated in high percentages, whereas only the control seeds without a valve germinated. Scanning electron micrographs showed that the hyphae penetrated, cracked, and eroded the funicular envelope of exhumed seeds.

1. Introduction

The genus *Opuntia* has 181 species and numerous varieties [1]. In the subfamily Opuntioideae, a hard funicular envelope completely encloses the seed [2–4]. A study of the seed hardness of 400 *Opuntia* varieties growing in San Luis Potosi, Mexico, showed that pressures from 171 to 456 kgf were required to break these hard seeds [5]. In species such as *Opuntia tomentosa* [6–9] the hardness of the funiculus limits water uptake and thus germination of the immature embryo. To achieve germination, the funiculus of *Opuntia tomentosa* can be weakened in three ways: a valve may form naturally during burial [9], the funiculus may be cracked through exposure to high daytime temperatures [9], or the funiculus may be eroded or cracked by microorganisms [8]. The valve is a region located in the funicular flanks, close to the micropyle; this is the site where the radicle protrudes naturally. This valve may be artificially removed to enhance germination.

Many studies relate the presence of microorganisms with deleterious effects on seeds [10]. However, the seed coat's tannins or other growth-inhibiting substances may serve as a barrier against the penetration of the seed by these. Additionally, the seed coat, formed by testa and tegmen, protects the seed's embryo from dehydration caused by temperature and humidity fluctuations [11, 12]. Therefore, most of the fungi observed to grow on intact, healthy seeds are saprophytic [13, 14] and even elicit seed germination, as in the impermeable and hard seeds of *Albizia julibrissin* and the achenes of *Rosa corymbifera* [15, 16]. It has been reported that in *O. streptacantha* fungi affect the germination in a species-specific manner; beacause the fungi weaken the testa [17, 18]. Nonetheless, germination in *O. streptacantha* is probably favored by the effects of fungi on the hard funiculus rather than on the testa.

To clarify how fungi enhance the germination of *Opuntia* seeds, we examined the effects of the presence of fungi on seeds and embryos via the following methods: (1) seeds stored in the laboratory and seeds that had previously been buried in the soil for five months were germinated in two ways, with and without the valve; (2) micrographs of both groups of seeds were used to identify any damage of the

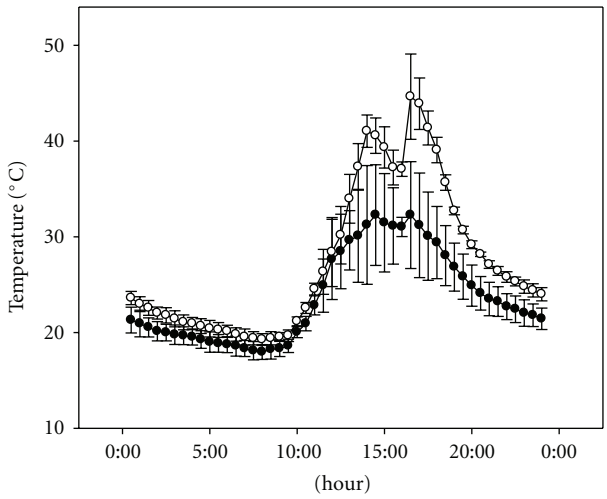


Figure 1: Daily soil temperature (mean ± S.D.; $n = 3$) in (∘) open sites (deprived of vegetation in the dry season) and (•) closed sites (beneath the canopy of perennial vegetation) in the Reserva Ecológica del Pedregal de San Ángel, Mexico.

funicular envelope by fungi during the seeds' residence in the soil; (3) the exhumed and laboratory-stored seeds and their isolated embryos were also placed in culture medium to promote the development of the fungi, which were isolated and subsequently identified.

2. Materials and Methods

2.1. Species and Study Site. Opuntia tomentosa S.D. (Cactaceae, subfamily Opuntioideae) is a perennial arborescent wild plant native to the arid zones of the Central Mexican Plateau [18]. In the southern region of the Mexico basin, this species grows in the volcanic zone known as Reserva Ecológica del Pedregal de San Ángel (REPSA, 19°19′N, 99°11′W). This species is characteristic of the xerophilous shrubland growing on shallow soils [19, 20]. The rainfall in this area is markedly seasonal, and the daily temperature fluctuation at the site is considerable because of the site's elevation (2250 m a.s.l) [7]. The seeds were collected from mature fruits at the site in December 2006. The soft funicles were manually separated from the seeds by washing them with tap water. The seeds were air-dried in darkness and stored at room temperature at 21 ± 1.78°C and 38.7 ± 6.3% relative humidity, inside glass containers.

2.2. Seed Burial. Portions of the seed lot were placed in six nylon net bags (25 × 25 cm) with 20 g per bag. Three bags were buried 5 cm deep in the soil for six months at two sites in the REPSA: a closed site and an open site. The closed site was located beneath the canopy of perennial vegetation (shrubs and trees); the open site was located in an area deprived of vegetation during the dry season; in the rainy season, this site is covered by grasses and annual Dicotyledoneae. The seeds remained in the soil five months from the time of collection (dry season) to the beginning of the following rainy season (from December 2006 to May 2007). After exhumation, the seeds were taken from the bag and dried in a dark room (23–25°C, 20–50% relative humidity) for a week and then stored in glass containers. During the burial period, the soil temperatures at a depth of 5–7 cm were recorded with a HOBO (model H01-001-01, Onset Computer Corporation, Pocasset, MA, USA); these results are shown in Figure 1.

2.3. Seed Germination. Laboratory-stored and exhumed seeds were treated with a 0.2% fungicide solution, Captan 50 ([*cis*-N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide], AGM, Mexico). After exhumation, the valve was removed from some of these seeds using a needle. Three replicates of 30 seeds each of laboratory-stored and exhumed seeds, with or without a valve, were sown on Petri dishes containing 1% agar water. The plates were placed inside growth chambers (Lab-Line Instruments, Inc., 844, Il, USA) at 25°C or at 25/35°C (18/6 h) under white fluorescent light (cool-white lamps, F20T12/CW, Sylvania, 20W), with a 12 h day^{-1} photoperiod. The radicle emergence was recorded every other day.

2.4. Scanning Electron Microscope Studies. The seeds were observed at the following different ages: (a) immediately after collection, (b) after 2 years of laboratory dry storage, (c) after 5 months of burial, and (d) after 19 months of laboratory dry storage after exhumation (24 months old). Before observation, the seeds were washed with tap water, air-dried, and fixed in FAA solution (formaldehyde 10%, ethanol 50%, acetic acid 5%, and distilled water 35%) [21]. The seeds were subsequently dehydrated in an ethanol-graduated series and dried with liquid CO_2 in a critical-point dryer (Balt-Tec CPD 030). The samples were mounted on aluminum stubs using a carbon double tape and coated with gold with a sputter coater (Desk II, Denton Vacuum Inc., Moorestown, NJ, USA). Observations were made with a scanning electron microscope (JSM-35, Electron Optics Div., Medford, MA, USA).

2.5. Isolation and Identification of Fungi from the Seeds. A fraction of the laboratory-stored seeds and exhumed seeds were surface-sterilized by immersion in a sodium hypochlorite solution (2%) for five minutes and subsequently sown on Petri dishes containing a culture medium of either potato-dextrose agar (PDA, 4%, Bioxon, Mexico) or water agar (2%) (WA, DIFCO, USA). The second fraction of these seeds was not disinfected, but it was washed with sterilized distilled water. If present, the fungi associated with the seed coat of nondisinfected and disinfected seeds and the fungi associated only with the embryos of the disinfected seeds were isolated and cultured from both laboratory-stored and exhumed seeds (from both burial sites). The embryos were extracted with a sterile needle after the seeds had been cut transversely with a sterilized scalpel. All of these procedures were performed under aseptic conditions inside a laminar flow cabinet (LABCONCO, Kansas City, MO, USA). In all experimental cases, three replications with 25 seeds each were used.

The seeds and embryos were incubated on Petri dishes containing PDA or water agar (2%). The dishes were

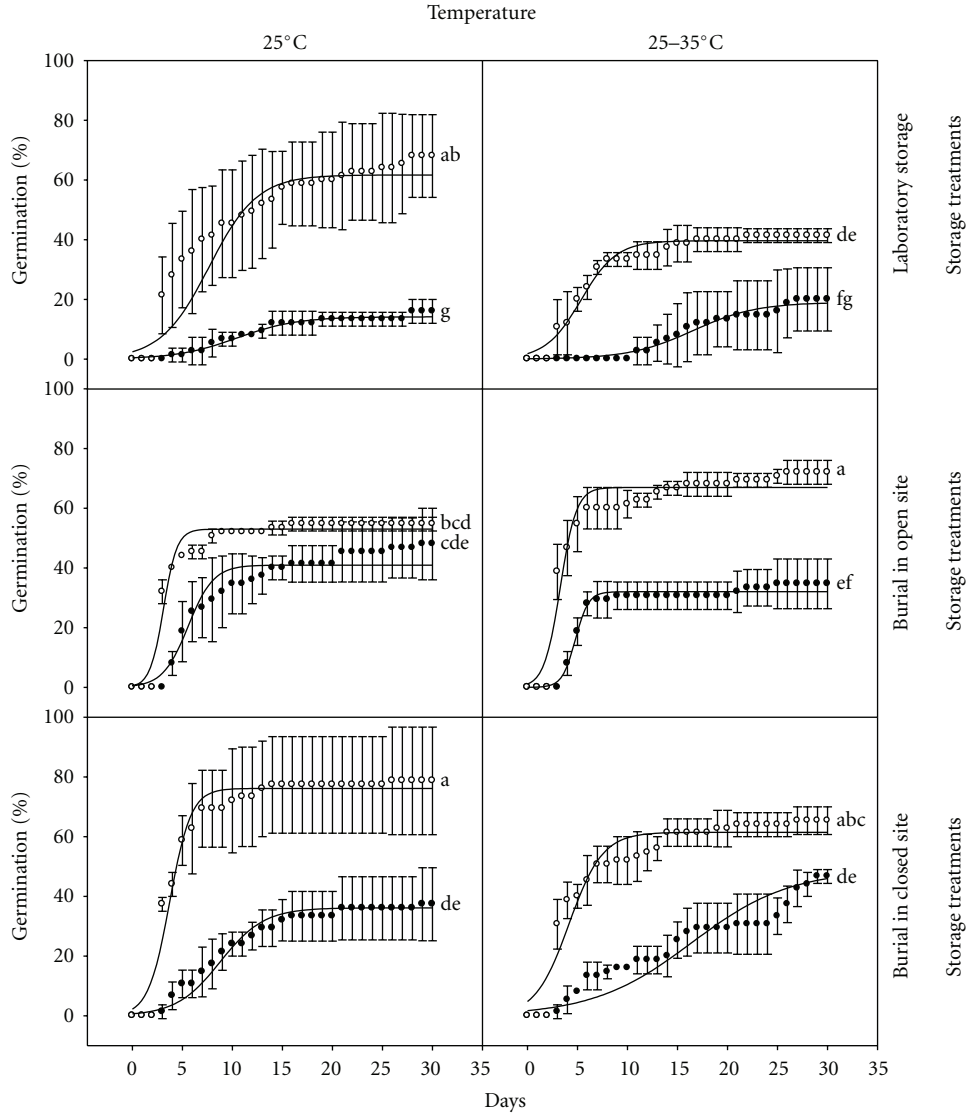


Figure 2: Effect of burial, temperature and valve removal on cumulative germination percentages of *Opuntia tomentosa* seeds (mean ± S.D.; $n = 3$). (•) Seeds with a valve, (∘) seeds without a valve. Letters in the figure indicate statistical comparisons.

incubated in an oven (Felisa, Mexico) at 25–27°C for 15 d. The plates were observed every other day. The presence or absence of fungi was determined. In some cases, the fungi found in the culture medium were isolated on PDA plates to obtain pure cultures and subcultivated twice. For the identification fungi were stained with Cotton blue stain and mounted on permanent slides with Elmer's White Glue (polyvinyl alcohol). The macromorphology of the fungi was observed with a stereoscopic microscope (Carl Zeiss Stemi DV4). The micromorphology was observed with an optical microscope (Olympus CX21; Olympus, Tokyo, Japan) with 40x and 100x objectives.

2.6. Statistical Analyses. Cumulative germination percentages were arcsine transformed and fitted to the sigmoid function $y = (\exp(-a/x) \times b/(1 + c) \times (\exp(d \times (e - x))))$ (Susana Orozco, personal communication) using Table Curve 2D, v. 3 software (AISN Software, Chicago, IL, USA). When the variances were homogeneous, the maximum germination and lag time (the time between sowing and the initiation of the germination) were compared using a three way ANOVA. LSD tests were used to perform multiple comparisons. If the data did not satisfy the requirements of normality and homoscedasticity, comparisons were made using the Kruskal-Wallis test [22]. Visual comparisons were made with box-and-whiskers plots ($P \leq .05$) using StatGraphics v. 5.0 (Statistical Graphics Corporation, Englewood Cliffs, New Jersey, USA).

3. Results

3.1. Seed Germination. Burial and valve removal significantly increased the seed germination percentages ($F_{(2,35)} = 14.50$, $P = .0001$; $F_{(1,35)} = 75.48$, $P = .0001$, resp.), with a significant interaction among the burial treatment, valve removal, and temperature variables

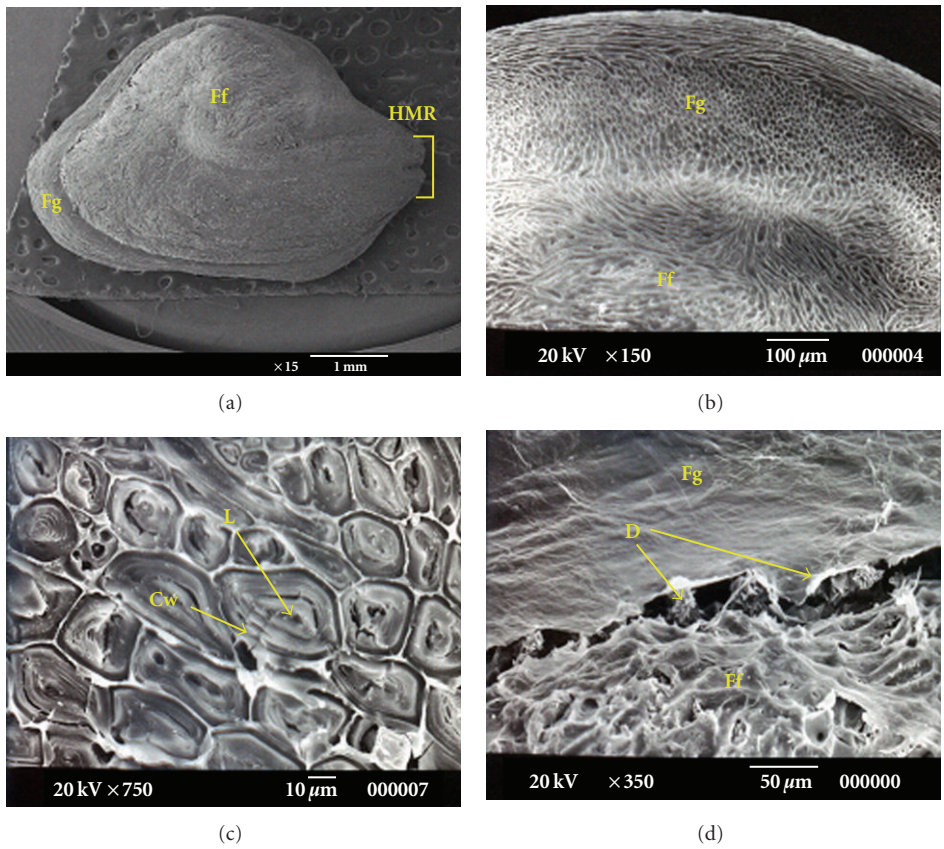


Figure 3: Micrographs of *Opuntia tomentosa* seeds recently taken from the fruit and washed with water: (a) whole seed; (b) fibers of the funicular seed cover; (c) funicular cell walls in a transversal cut; (d) druses in the funicular seed cover and the remains of the soft funicle. Cw: cell walls; D: druses, Ff: funicular flanks, Fg: funicular girdle, HMR: hilum-micropylar region, L: lumen.

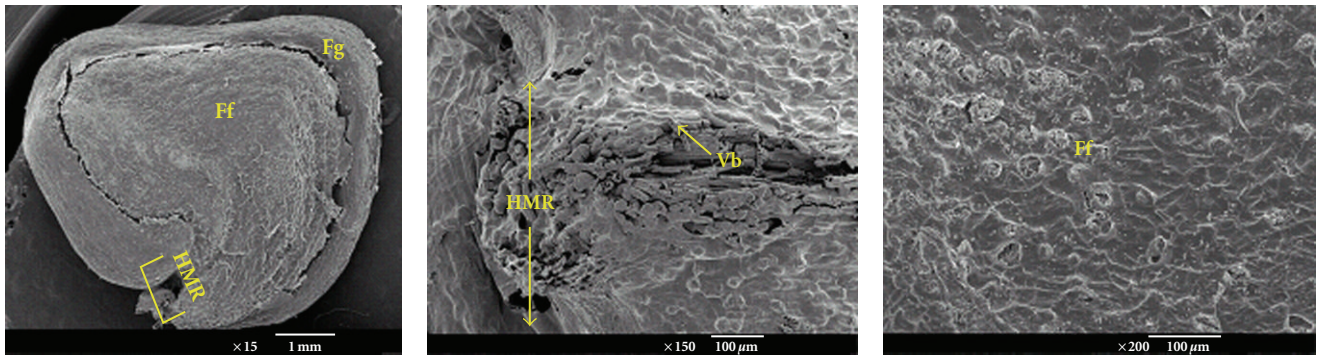
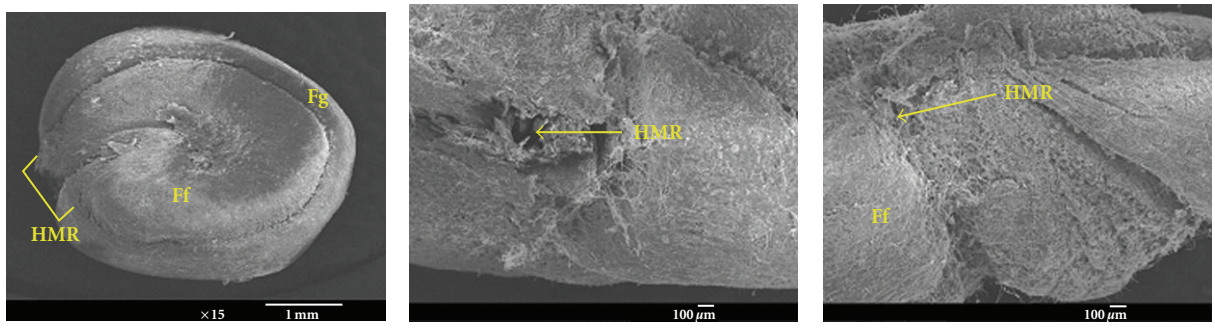


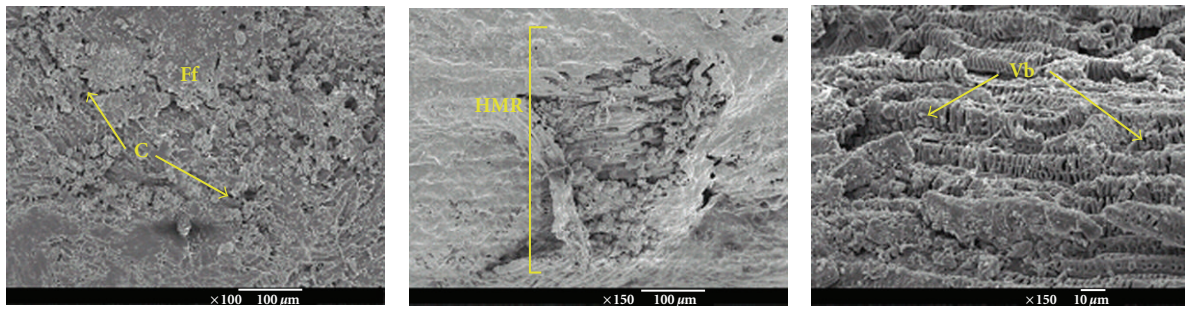
Figure 4: Micrographs of *Opuntia tomentosa* seeds after two years of laboratory storage: (a) whole seed showing the funicular girdle separated from the remaining funicular seed cover; (b) cracks in the hilum-micropylar region; (c) degradation of the surface layers (cuticle) of the funicular flanks. Ff: funicular flanks, Fg: funicular girdle, HMR: hilum-micropylar region, Vb: vascular bundles.

($F_{(2,35)} = 8.17$, $P = .002$). Independent of germination temperature, the laboratory-stored seeds lacking a valve germinated at higher percentages than the seeds with a valve (Figure 2). At 25°C and without the valve, the seeds stored in the laboratory showed high germination percentages similar to those of the buried seeds. At alternating temperatures, the seeds stored in the laboratory and the seeds that germinated without a valve had lower germination percentages than they did at 25°C. These germination percentages were similar to those of the buried seeds with a valve that were germinated at alternating temperatures. The seeds buried at the open site and germinated at 25°C had similar germination percentages, either with or without a valve. The laboratory-stored seeds with a valve showed the lowest germination percentages at both temperatures.



(a) (b) (c)

Figure 5: Micrographs of *Opuntia tomentosa* seeds exhumed after 5 mo of burial: (a) whole seed showing a slight separation of the funicular girdle; (b) deep degradation in the hilum-micropiliar zone and in the surface layers of a seed buried at the open site; (c) deep degradation in the hilum-micropiliar zone and in the surface layers of a seed buried at the closed site. Ff: funicular flanks, Fg: funicular girdle, HMR: hilum-micropylar region.



(a) (b) (c)

Figure 6: Micrographs of *Opuntia tomentosa* seeds exhumed after 5 months of burial and laboratory storage for 2 years. (a) Deep degradation of the funicular flanks in seeds buried at the open site, (b) cracks in the hilum-micropylar region, (c) degraded and eroded funicular envelope exposing the vascular bundles. C: cracks, Ff: funicular flanks, Fg: funicular girdle, HMR: hilum-micropylar region, Vb: vascular bundles.

Temperature alone did not affect the lag time ($F_{(1,35)}$ = .49, P = .490). Burial and valve removal significantly reduced the lag time ($F_{(2,35)}$ = 31.43, P = .001; $F_{(1,35)}$ = 51, P = .0001, resp.). There were also significant interactions between the three factors ($F_{(2,35)}$ = 5.08, P = .014) (Figure 2). The longest lag times were observed in the laboratory-stored seeds sown with a valve. These lag times were significantly higher at alternating temperatures than at a constant temperature. The valve treatments did not have a significant effect on the lag times of the buried seeds.

3.2. Scanning Electron Microscope Studies. Storage and burial produced changes in the funicular envelope of *O. tomentosa* seeds. Figure 3(a) shows the seeds taken directly from the fruit. The funicular surface contains lignin fibers (Figure 3(b)). The thick cell walls of the fibers may be observed in the transverse section (Figure 3(c)). When the funicular girdle began to separate from the remaining funicular envelope, druses were also observed beneath the surface (Figure 3(d)). A substantial separation of the funicular girdle was observed in seeds stored for two years in the laboratory (Figure 4(a)). Some fractures and the exposure of the vascular bundles were observed in the hilum-micropylar zone (Figure 4(b)). No fractures were found in the funicular flanks; however, there was a slight degradation of the cuticle (Figure 4(c)). A slight separation of the funicular girdle occurred in the seeds buried at the open site (Figure 5(a)). A deep degradation was observed in the funicular flanks of the seeds buried at the open and closed sites (Figures 5(b) and 5(c), resp.); the degradation was present mainly in the hilum-micropylar region.

The separation of the funicular girdle was also observed in seeds buried for five months in the open and closed sites (micrographs not shown). The funicular flanks of the seeds buried at both sites showed a deep degradation. Cracks and a wide, deep degradation of the cuticle were observed in the funicular flanks of the seeds buried at the open site (Figure 6(a)) and in the hilum-micropylar region of the seeds buried at the closed site (Figure 6(b)). In the exhumed seeds from both sites, the degradation of the funiculus exposed and eroded the vascular bundles (Figure 6(c)). A high density of filamentous structures covering the surface of the funiculus was observed in the seeds buried for five months. The tubular structures with homogeneous form were considered to be hyphae (Figures 7(a) and 7(b)). Other accompanying structures had the appearance of conidiophores and spores (Figures 7(c) and 7(d)).

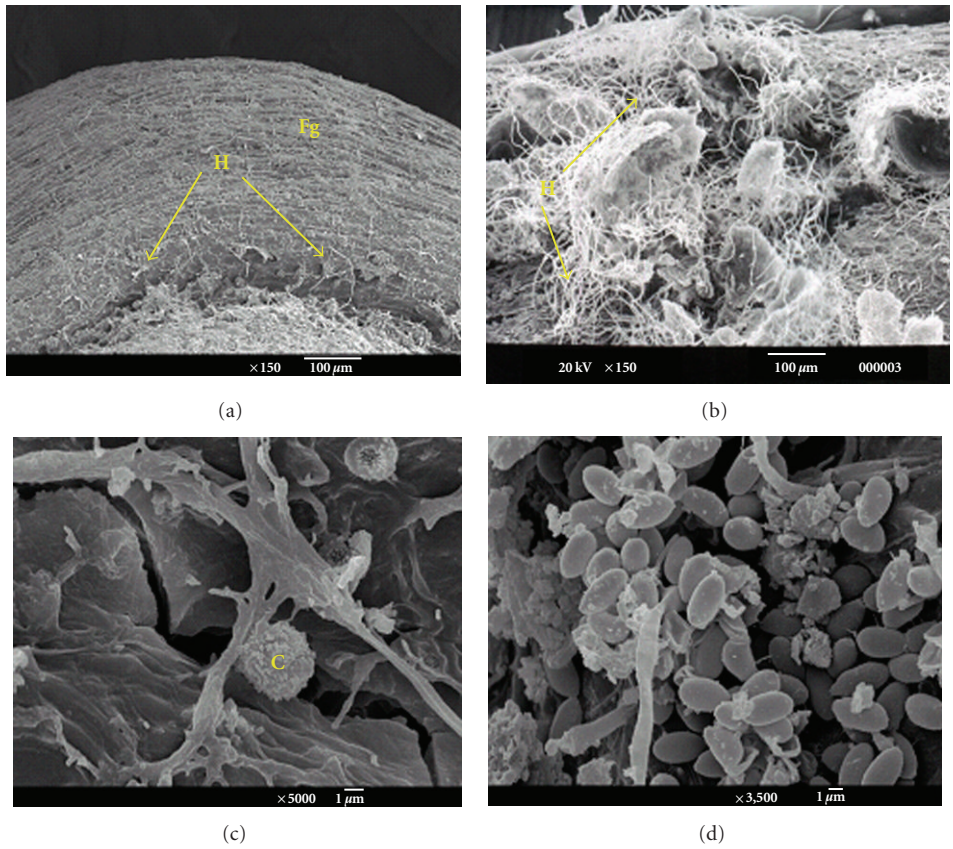


Figure 7: Micrographs of microorganisms on the funicular cover of *Opuntia tomentosa* seeds exhumed after 5 months of burial: (a) hyphae on the funicular envelope; (b) massive growth of mycelia on the funicular envelope; (c) hyphae and conidiophores, (d) spores. C: conidiophores, Fg: funicular girdle, H: hyphae.

3.3. Isolation and Identification of Fungi from the Seeds. After 10 days of incubation, 3 species of *Penicillium* were found on the nondisinfected, laboratory-stored seeds. In the embryos of disinfected seeds and in the disinfected seeds that were laboratory-stored, no organisms were found in either culture medium. The seeds that were buried at the open site and subsequently disinfected revealed the presence of the following 4 genera of fungi: *Basidiobolus*, *Gelasinospora*, *Phoma*, and *Trichoderma*. The nondisinfected seeds buried at the open site showed the presence of *Circinella* sp., *Phoma porum*, and *Rhizopus stolonifer*. The disinfected seeds buried at the closed site showed the presence of *Fusarium* sp., *Nigrospora* sp., and *Rhizopus stolonifer*. The nondisinfected seeds buried at the closed site showed the presence of only 1 fungal genus, *Aureobasidium* sp.

4. Discussion

The laboratory-stored seeds of *O. tomentosa* did not germinate and temperature fluctuation did not improve the germination percentage even if the valve was removed; one exception was observed in the seeds buried in the open site (Figure 2). The valve can be removed easily from seeds that have been buried. However, manual valve removal was required to achieve the highest germination percentages in the exhumed seeds. Additional structural changes in the funicular envelope during dry storage were observed in the micrographs of exhumed seeds after two years of laboratory storage, which may explain the high germination percentages reported by Olvera-Carrillo et al. [8] in stored exhumed seeds.

In the soil seed bank, valve formation, the weakening of the funiculus, and the loss of dormancy should involve several steps and the action of several soil factors, as suggests the field germination reported by Olvera-Carrillo et al. [7]. The photomicrographs revealed a profuse growth of mycelia on the funicular envelope of the exhumed seeds (Figure 7(b)), indicating the presence of an initial successional step by the soil fungi that are able to utilize readily available sugars [23]. Fungi such as *Aureobasidium*, *Basidiobolus*, *Circinella*, and *Rhizopus* may acquire nutrients from the remains of the enriched tissue, where seeds are imbibed into the fruit [9].

It has been proposed that fungal development is a requirement for the seed germination of *O. leucotricha* and *O. streptacantha* [17, 18]. In this study, 11 genera of fungi were found growing on the seeds of *O. tomentosa*. Four genera and/or species even grew on exhumed, disinfected seeds. This growth probably occurred because the spores or hyphae were protected by the deep layers of the thick funiculus and germinated when the populations of the superficial fungal

genera (*Aureobasidium*, *Circinella* sp., *Phoma pomorum*, and *Rhizopus stolonifer*) were depleted by the sodium hypochlorite solution. Among the five species growing on exhumed, nondisinfected seeds, only *Phoma porum* was shared with the fungi growing on disinfected seeds.

Rhizopus stolonifer, *Gelasinospora*, *Basidiobolus*, *Nigrospora* sp., *Fusarium* sp., and *Trichoderma* also grew on exhumed, disinfected seeds. Different degrees of cellulolytic activity have been demonstrated in filamentous-Ascomycota [24, 25]. These fungi are characteristic of a second successional step during fungal colonization [23], and they may play an important role in the weakening of the funicular cover. For example, *Rhizopus* sp. increases seed germination of *Thelocactus hexahedrophorus* [26]. In contrast, no fungi grew in the disinfected, laboratory stored seeds. *Alternaria* and *Penicillium* spp. were only present in the nondisinfected seeds, suggesting that these fungy were acquired during the process of handling before laboratory storage.

Differences in the mycoflora found on the laboratory-stored and exhumed seeds suggest that fungal colonization of *O. tomentosa* seeds occurs mainly in the soil. Nevertheless, no fungal growth was found in any of the embryos isolated from seeds. This result could indicate that fungi weaken the funicular envelope but do not injure the *Opuntia* seed coat, supporting the role that the seed coat plays in protecting the embryo. In *O. tomentosa*, the seed coat (the testa and the tegmen) is located beneath the hard funicular envelope; this coat contains a large level of tannins (phenolic compounds) [9], which plays a protective role in the seeds of many species, especially for seeds that possess hard seed covers [12, 27]. The presence of the seed coat extends *Opuntia* seed viability in the laboratory and in the soil.

The erosion of the funicular surface by fungal activity on the funicular cover of *O. tomentosa* seeds is evident in the micrographs obtained in this research. The observed damage to the funicular surface resembles the forms of damage produced by *Fusarium*, *Pythium* and *Rhizoctonia* in the seeds of *Albizia julibrissin* [15]. However, the germination percentages in this species were relatively low compared with those found for *O. tomentosa* in the current study.

In the REPSA, *O. tomentosa* seeds are dispersed by zoochory in the dry season (winter). In this season, both the heterogeneous volcanic substrate and the heterogeneous plant cover offer a wide number of microenvironments that may either favor or inhibit the colonization of the seeds by fungi. However, the germination percentages of the seeds buried in the open sites and in the closed sites did not differ significantly in this study or in the work by Olvera-Carrillo et al. [7, 8]. In this study we found differences in the mycoflora present in the disinfected and nondisinfected seeds exhumed from both burial sites. Together, these findings suggest that the funicular envelope is weakened by a combination of soil factors, such as moisture and temperature fluctuations (see Figure 1). For example, *Gelasinospora* is resistant to high temperatures while *Penicillium* is not [28]. The variety of germination responses in buried seeds with and without a valve, the slow velocity of lignin decomposition, and the limited number of lignin-decomposing fungi species in the soil [23] may partially explain why germination of the physiologically dormant *O. tomentosa* embryos is extended in the field for at least two years [8].

Acknowledgments

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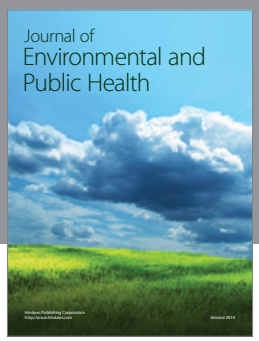

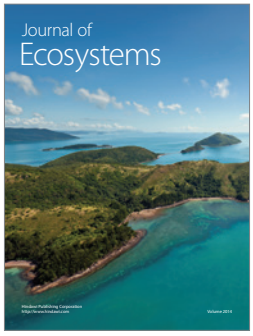

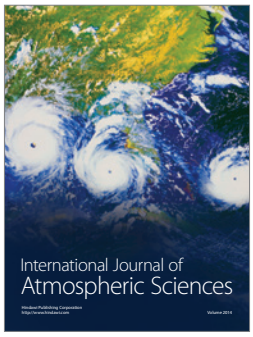

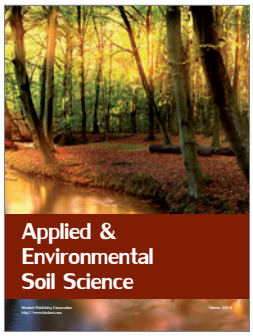

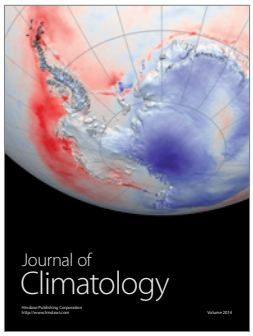

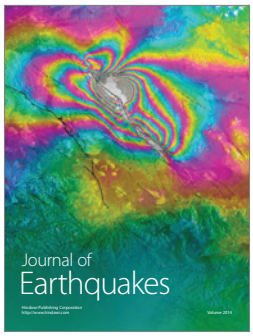

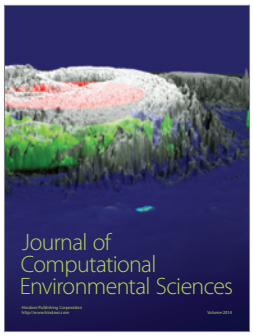

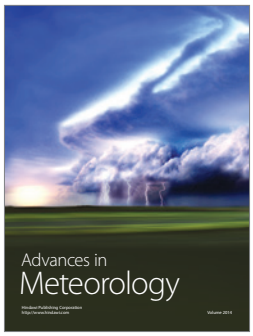

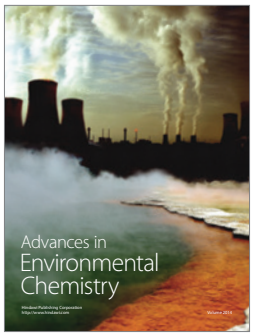

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