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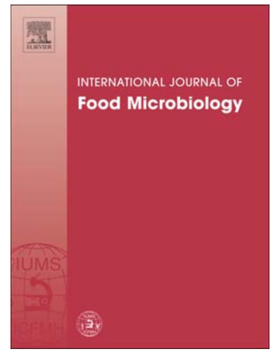

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The influence of environmental factors on growth and interactions between *Embellisia allii* and *Fusarium oxysporum* f. sp. *cepae* isolated from garlic

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Abstract

Embellisia allii results in the formation of a bulb canker and black sooty on the surface of different alliums and it has been frequently detected on garlic bulbs together with the spoilage fungus, *Fusarium oxysporum* f. sp. *cepae*, which causes bulb basal plate rot. In this study, the influence of water activity (a_w) and temperature on mycelial growth of *E. allii* and *F. oxysporum* f. sp. *cepae*, conidial size and sporulation of *E. allii*, interactions between *E. allii* and *F. oxysporum* f. sp. *cepae*, Index of Dominance (I_D), and *in situ* virulence on garlic were examined. Mycelial growth of *E. allii* was optimal (5.97 mm/day) at 0.995 a_w and 25℃, slower at 30℃. However, almost no growth occurred at 0.937 a_w/30℃. *F. oxysporum* f. sp. *cepae* grew faster than *E. allii*, (6.3-7.4 mm/day) at 30℃. Interactions between *E. allii* and *F. oxysporum* f. sp. *cepae* were influenced by aw and temperature**.** Sporulation of *E. allii* was more abundant on PDA than on MEA, especially at high a_w (0.995) and low temperature (20℃), but almost no sporulation occurred at 30℃ regardless of nutritional medium or a_w level. The spore length of *E. allii* was longer on PDA than MEA, and was significantly influenced by water availability. *F. oxysporum* f. sp. *cepae* was competitive against *E. allii* and had a higher I_D value in comparison with *E. allii* especially at a higher temperature (30℃). *In situ* virulence tests showed that *E. allii* was weakly virulent on the garlic bulb cloves while that of *F. oxysporum* f. sp. *cepae* was highly dependent on a_w.

Keywords: Embellisia allii, *Fusarium oxysporum* f. sp. *cepae*, Water activity (a_w), Temperature, Interactions

1. Introduction

Garlic (*Allium sativum* L.), an important bulbous plant, is used mainly as a seasoning for foods, and increasingly as an herbal medicine and pesticide in organic farming (Pitman and Burt, 2001). Garlic can be damaged by a number of soil inhabiting species and/or storage fungi (Stadnik et al., 1994). When Soares and Kurozawa (1998, 1999) examined samples of garlic bulbs from Brazil, the most prevalent genera were *Fusarium* (88.6%), *Penicillium* (77.1%) and *Aspergillus* (9.3%), and the most dominant species was *Embellisia allii* (Campan.) Simm. (2.5%).

F. *oxysporum* f. sp. *cepae* (Hanz.) Snyder & Hansen can cause basal plate rot, which is characterized by a firm pinkish brown rot covered with whitish mycelium, and less frequently foliar disease (Schwartz and Mohan, 2006). This occurs wherever alliums such as onion, garlic, shallot, chive and leek are cultivated; however it has also been frequently observed on garlic bulbs (Crowe et al., 1986; Everts et al., 1985; Matuo et al., 1986). Garlic bulb canker caused by *E. allii* has been reported in many countries including the USA (Simmons, 1971; David, 1991; Farr & Rossman, 2009), Canada (Corlett, 1996), Japan (Taniguchi et al., 1994) and Korea (Lee et al., 2002). *E*. *allii* attacks the outer scales of the garlic bulb, producing a black soot that covers the surface during cultivation as well as after storage. In mature bulbs, this disease affects their appearance and hence their market value.

A previous survey conducted in Korea revealed that bulb canker and basal rot disease existed not only on newly harvested bulbs from different cultivation areas but also on polyfilm-wrapped cloves sold in the markets (Lee et al., 2002). Previously, Taniguchi et al. (1994) reported that the garlic bulb canker caused by *E. allii* is a minor disease. However, we have observed that losses due to decay of garlic were occasionally severe. There have been no reports on the growth and sporulation of *E. allii* under different environmental conditions. In addition, little information is available about the co-occurrence of *E. allii* and *F. oxysporum* f. sp. *cepae* on garlic bulbs and the interactions that may occur between those two species during storage. Dominance and colonization on different substrates may be considerably influenced by environmental or biological factors such as water activity, niche temperature, and inter-specific interactions (Magan and Lacey, 1984; Lee and Magan, 1999; Lee and Magan, 2000; Magan and Aldred, 2007; Giorni et al., 2008). Such information may be critical in understanding the conditions that enable individual fungi to become dominant in the garlic niche, especially post-harvest.

The objectives of this study were to determine (a) the effect of water activity (0.937, 0.971, and

0.995 a_w) and temperature (20, 25, and 30℃) on *in vitro* mycelial growth, sporulation and spore size of *E. allii*, *and F. oxysporum* f. sp. *cepae,* (b) the relative competitiveness of these fungi and (c) *in situ* pathogenicity on garlic.

2. Materials and methods

2.1. Fungal species

Diseased garlic bulbs were collected directly from cultivation areas or storehouses of Seosan and markets of Cheongyang, Suwon, and Daejeon, Korea. Spoilage fungi were isolated from bulb cankers and basal rot lesions of stored garlic. Based on morphology and sequence analyses of the internal transcribed spacer (ITS) region of 18S ribosomal DNA, the fungi were confirmed to be *E. allii*, and *F. oxysporum* f. sp. *cepae*, respectively. The molecular characterization of the strains was performed using previous methods (Lee et al., 2005; Lee et al., 2006). When the sequence of the isolates was compared with related species retrieved from GenBank, the intra-specific DNA homologies were between 99-100% identical (data not shown). All strains have been deposited in the EML herbarium (Chonnam National University, Gwangju, Republic of Korea) as *E. allii* HBL-EA01 to HBL-EA03, *F. oxysporum* f. sp. *cepae* FO-HBL05 and FO-HBL06), respectively.

2.2. Media

The basal media were (a) 2% malt extract agar (MEA, Difco) at pH 5.5 and (b) 4% potato dextrose agar (PDA, Difco). The water activity (a_w) of the basal MEA medium was 0.995, which was modified to 0.937 and 0.971 by using glycerol+ water solutions. MEA + 2% ground garlic extract (MEGA) was also used as a semi-natural medium in this study. The a_w of media was measured using a Novasina ICII (Novasina AG, Zurich, Switzerland).

2.3. Effects of water activity and temperature on mycelial growth, sporulation and spore size

Stock cultures of test species were grown on MEA at 25℃ and used as inocula. Growth rates of *E*. *allii* and *F. oxysporum* f. sp. *cepae* were determined by transferring mycelial plugs (5 mm in diameter) from the growing margin of stock cultures in Petri dishes (90 x 15 mm in diameter) containing MEA, PDA and MEGA. After inoculation, plates with the same a_w were sealed in polyethylene bags and incubated at 20, 25, and 30℃ in darkness for up to 10 days. Mycelial growth was recorded periodically by measuring the diameter of the colony in two directions at right angles to each other.

Spore production was determined after 14 days incubation by placing entire colonies grown on media into flasks with a measured volume of distilled water. All the experiments were carried out with at least three separate plates per treatment. Five ml of distilled water was added to each plate and spores were dislodged from the plates using a soft brush. Using a haemocytometer, the spore length, width, and septation was determined of 30-50 spores for each treatment and replicate (n=3).

2.4. Interactions between E. allii and F. oxysporum f. sp. cepae

Dual culture tests were carried out under different a_w and temperature regimes by inoculation of the species (10 mm diameter agar plugs) 4 cm apart. The diameter of the growing colonies was measured daily as described previously (Magan and Lacey, 1984; Lee and Magan, 1999; Lee and Magan, 2000). The macroscopic interactions between mycelia in dual cultures were examined and each species given a score based on mutual intermingling (1-1), mutual antagonism (2-2), mutual antagonism at a distance (3-3), dominance of one species upon contact (4-0) and dominance at a distance (5-0) according to Magan and Lacey (1984). An Index of Dominance (I_D) was used to measure the ability of a species to dominate under a particular set of treatment conditions. The scores for each species were added to obtain the overall I_D.

2.5. In situ virulence test on garlic

The ability (= *in situ* virulence) of *E. allii* and *F. oxysporum* f.sp. *cepae* to infect garlic bulbs was evaluated under different relative humidities in plastic boxes adjusted to different a_w levels. The RH in the boxes was adjusted to 0.999 a_w (= 99.9% RH), 0.971 (= 97% RH) and 0.937 (= 93.7% RH). For inoculation, spores (10^5/ml in *E. allii* and 10^6/ml in *F. oxysporum* f. sp. *cepae*) were prepared and hand sprayed on the garlic bulb cloves (either surface wounded with a sterile needle, or non-wounded). The a_w conditions and experimental conditions were as detailed in Beecher *et al*. (2001). Their virulence was determined by the symptom level (+++, very virulent; +, slightly virulent, -, not virulent) on garlic at 25℃ after 12 days.

3. Results

3.1. Effects of water activity and temperature on mycelial growth, sporulation and conidial size

Figure 1 shows the effect of a_w on growth of *E. allii* on both a characterized artificial medium and on that containing garlic. This was faster at 0.995 a_w on the control substrate (MEGA without glycerol. There was no significant difference in growth of *E. allii* between the two media. Figure 2 shows the effect of a_w and temperature on growth of two strains of F. *oxysporum* f. sp. c*epae.* At 0.995 and 0.971 a_w, isolates FO-HBL05 and FO-HBL06 grew faster at 30°C than at lower temperatures. Regardless of temperatures, the FO-HBL06 isolate was more tolerant of water stress (0.937 a_w) than FO-HBL05.

Sporulation of *E. allii* was better on PDA than MEA, especially at a high a_w level (0.995 a_w) and at the lowest temperature tested (20℃, Fig. 3). Ho wever, almost no sporulation occurred at 30℃ regardless of media and a $_w$ used. Practically no sporulation occurred at 30℃ r egardless of media used. However, the conidial size *E. allii* conidia was affected by medium, a_w, and temperature (Table 1).

3.2. Interactions between E. allii and F. oxysporum f. sp. cepae

There was mutual inhibition between *E. allii* and *F. oxysporum* f. sp. *cepae* strains depending on the environmental conditions and medium. At 0.995 a_w, *E. allii* was inhibited more by strain FO-HBL06 than by FO-HBL05 at 25-30℃ in comparison wit h the control. Interaction patterns and the I_D values among *E. allii* and the mean scores of two *F. oxysporum* f. sp. *cepae* strains are shown in Figure 4. Generally, the two isolates of *F. oxysporum* f. sp. *cepae* were more competitive against *E. allii* and had a higher I_D value.

3.3. In situ pathogenicity of spoilage pathogens

Table 2 shows that the relative ability of *E. allii* and *F. oxysporum* f. sp. *cepae* to infect garlic bulbs was affected by water activity (=% relative humidity). *In situ* pathogencity of both decreased when the water availability was reduced (0.937 a_w), but *F. oxysporum* f. sp. *cepae* was more tolerant than *E. allii* to water stress. *E*. *allii* was unable to infect garlic bulbs when the water availability was 0.937 and 0.971 a_w.

4. Discussion

The present study has shown that besides *Penicillium* and *Botrytis,* decay fungi including *E*. *allii* and *Fusarium* species are also associated with the spoilage of garlic bulbs. Both these latter species have been frequently found with basal rot and clove rot fungus and in garlic bulbs packed in bags or stored for long periods at low temperatures. Before and after harvest, the garlic bulbs carry a wide range of fungi as spores or mycelia. These fungi will interact with each other and some will become more dominant than others. These interactions between species are influenced by many parameters; however, substrate (nutrient), water availability (a_w) and temperature are known to be the most important abiotic parameters that determine growth (Magan and Aldred, 2007; Pateraki et al., 2007). Interestingly, the ecophysiological characteristics of *E. allii* in a garlic bulb niche is important in understanding which spoilage fungus can be the dominant invader or how certain other spoilage species can be dominated by other interacting fungi. The present results showed that the preferential establishment of *F. oxysporum* f. sp. *cepae* might prevent *E. allii* or other important spoilage fungi such as *Aspergillus* and *Penicillium* from colonizing the same bulb of garlic. Among the isolates of *F. oxysporum*, there were differences in their ability to compete against *E. allii.* This was supported by the overall higher I I_D value.

Based on the virulence test, *E*. *allii* was weakly pathogenic in wounded garlic. This is in ore agreement with a previous study by Taniguchi *et al*. (1994). *E*. *allii* did not infect garlic bulbs under lowered a_w (0.937, 0.971 a_w), but only when water was freely available and under cooler conditions during longer term storage. Often wrapping and packaging with polyfilms barriers allow a buildup of water due to respiration and can promote discoloration and spoilage. *F*. *oxysporum* f. sp. *cepae* grew fast under warmer conditions. The optimum storage temperature for seed garlic is 10℃ with a humidity of 65-70%. Garlic cloves sprout most rapidly between 4.5-10℃ and thus prolonged storage at this tempera ture range should be avoided. *E. allii* was more sensitive to water and temperature stress than the isolates of *F. oxysporum* f. sp. *cepae*. Until recently, there have been little statistical data on the economic repercussions of the damage caused to garlic by *F. oxysporum* f. sp. *cepae* in Korea, but damage due to unfavorable storage has been reported to be severe (data not shown). *F*. *oxysporum* survives indefinitely in soil. Infection occurs through wounds or in the vicinity of old root scars at the base of the bulb. The disease favors soil temperatures in the range of approx. 13 to 32℃, with optimum temperatures ranging from approx. 26-27℃. Basal ro t is more prevalent in transplanted onions than in direct-seeded onions (UC IPM Online, 2004). The roots of plants infected early in the growing season can decay and die or turn yellow and wilt. The bulbs of plants infected later in the season may appear normal, but the inner neck tissue may feel soft and portions or all of the basal plate develop a firm dry rot, turn brown, and die. Under moist conditions, the diseased scales can develop white mold (UC IPM Online, 2004).

The present study has identified that these two fungi have different ecologically niches in which they are able to cause spoilage in garlic. This basic understanding of the post-harvest biology of these two spoilage fungi will be beneficial to design control strategies based on the weaker points in their life cycle.

Acknowledgements

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Table 1. Effect of water activity (a_w), temperature and medium on conidial dimensions (morphology) of *Embellisia allii* HBL-EA01

Condition	Conidial dimension		
	No. of septa	Spore width (μm)	Spore length (μm)
20℃			
PDA / basic	3.2±0.17[a]	13.4±0.35	30.4±0.79
MEA / 0.995 a_w	2.9±0.09	11.7±0.30	25.2±0.56
MEA / 0.971 a_w	1.9±0.15	12.5±0.36	21.2±1.09
25℃			
PDA / basic	2.2±0.21	13.0±0.39	22.0±1.02
MEA / 0.995 a_w	2.1±0.18	12.0±0.32	21.6±0.91
MEA / 0.971 a_w	2.0±0.20	11.5±0.45	20.6±1.02

[a]Mean ±SD value. At 30℃, no examination because of poor conidiation.

Table 2. *In situ* pathogenicity of strains of *E*. *allii* and *F*. *oxysporum* f. sp. *cepae* strains on garlic under different water activity conditions

Isolate	Water activity level			
	Control*	0.995	0.971	0.937
E. allii HBL-EA01	+**	+	-	-
E. allii HBL-EA03	+	+	-	-
F. oxysporum f. sp. *cepae* HBL05	++	++	+	-
F. oxysporum f. sp. *cepae* HBL06	++	++	+-	-

*Control: water saturated condition (without glycerol added), +**: slightly virulent, ++: highly virulent, +-: very slightly virulent, -: not virulent. The virulence on garlic was determined at 25℃ after 12 days.

Figures captions

Figure 1. Mycelial growth of *Embellisia allii* HBL-EA01 on malt extract agar (MEA) and malt extract galic agar (MEGA) under different water activity conditions (□–□, 0.995; □–□, 0.971; □–□, 0.937) at 25℃. The radii of all colonies were me asured after 6 days inoculation.

Fig. 2. Mycelial growth of *F*. *oxysporum* f. sp. *cepae* (strains FO-HBL05, FO-HBL06) on malt extract agar (MEA) under different water activity conditions (□–□, 0.995; □–□, 0.971; □–□, 0.937) at 20 (A), 25 (B) and 30℃ (C).

Fig. 3. Sporulation of *E*. *allii* HBL-EA01 on malt extract agar (MEA) and potato dextrose agar (PDA) at different water activity conditions (□–□, 0.995; □–□, 0.971; □–□, 0.937) and at 20 (A), 25 (B), and 30℃ (C). Bars represent standard error s of estimated parameters.

Fig. 4. Mean Index of Dominance (I_D) of *E*. *allii* HBL-EA01 when paired with two strains of *F*. *oxysporum* f. sp. *cepae* (FO-HBL05, FO-HBL06) on malt extract agar (MEA) under different water activities (A, 0.971; B, 0.995 a_w) at 20, 25, and 30℃ .

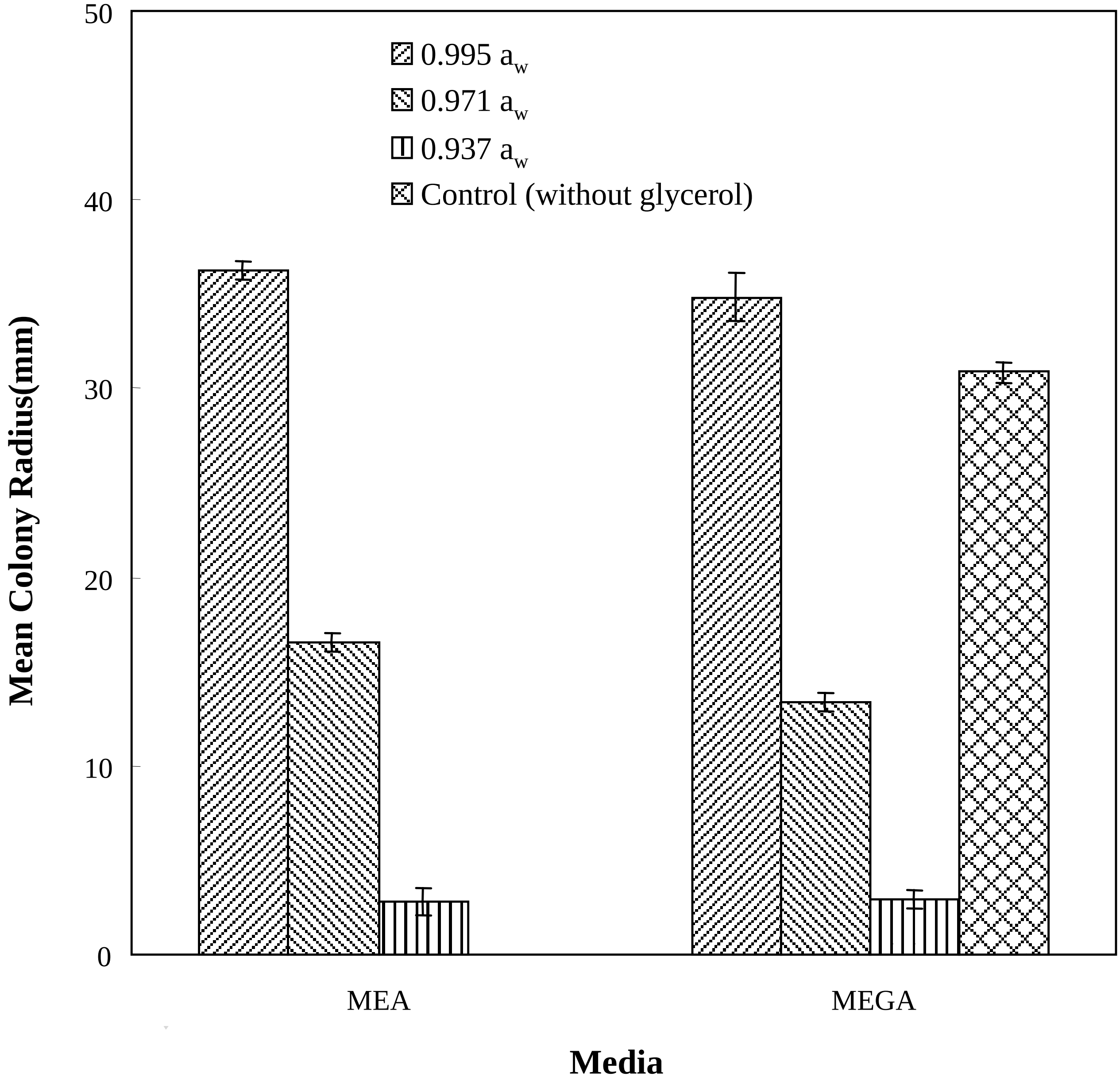

Figure 1. Lee and Magan

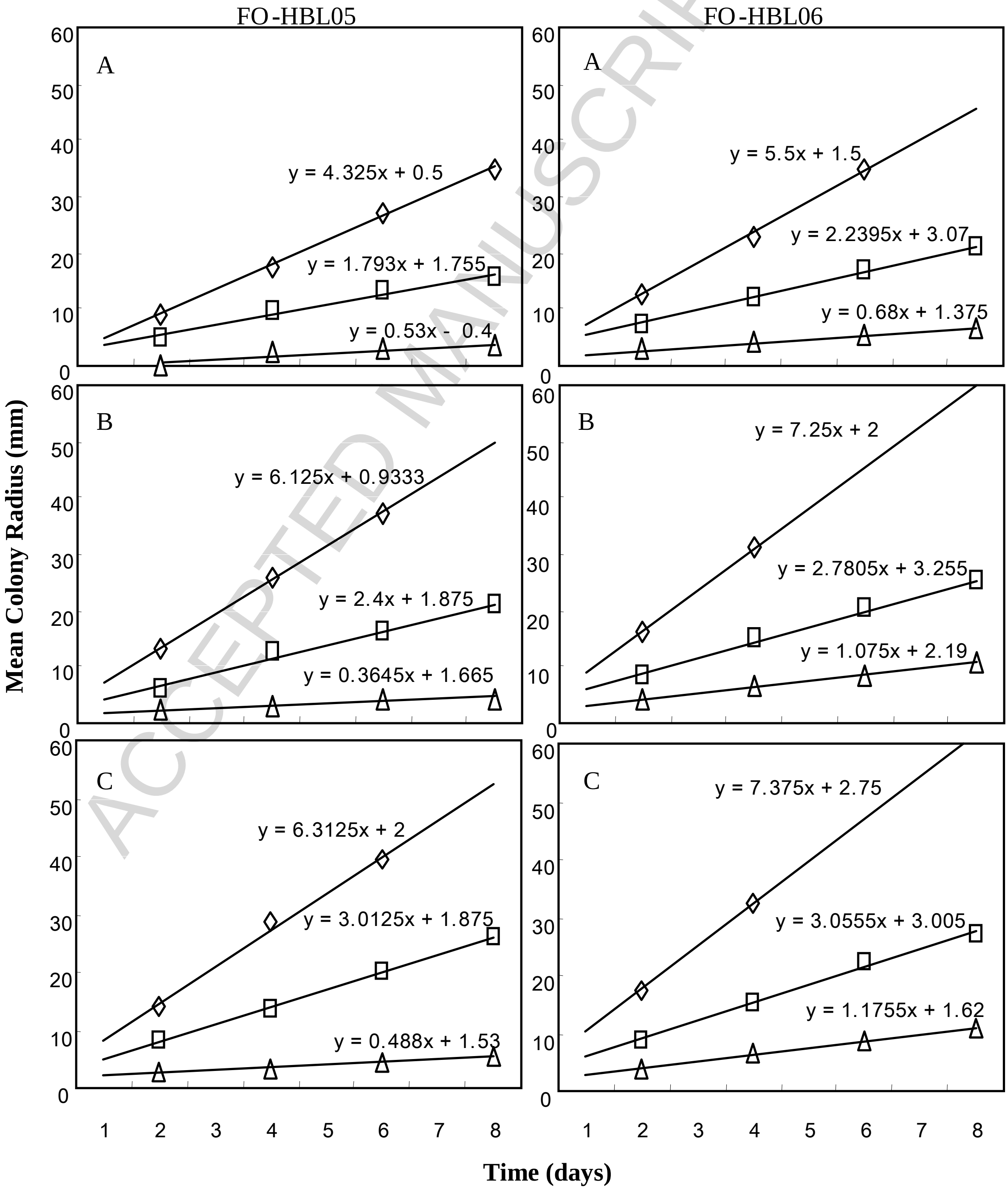

Figure 2. Lee and Magan

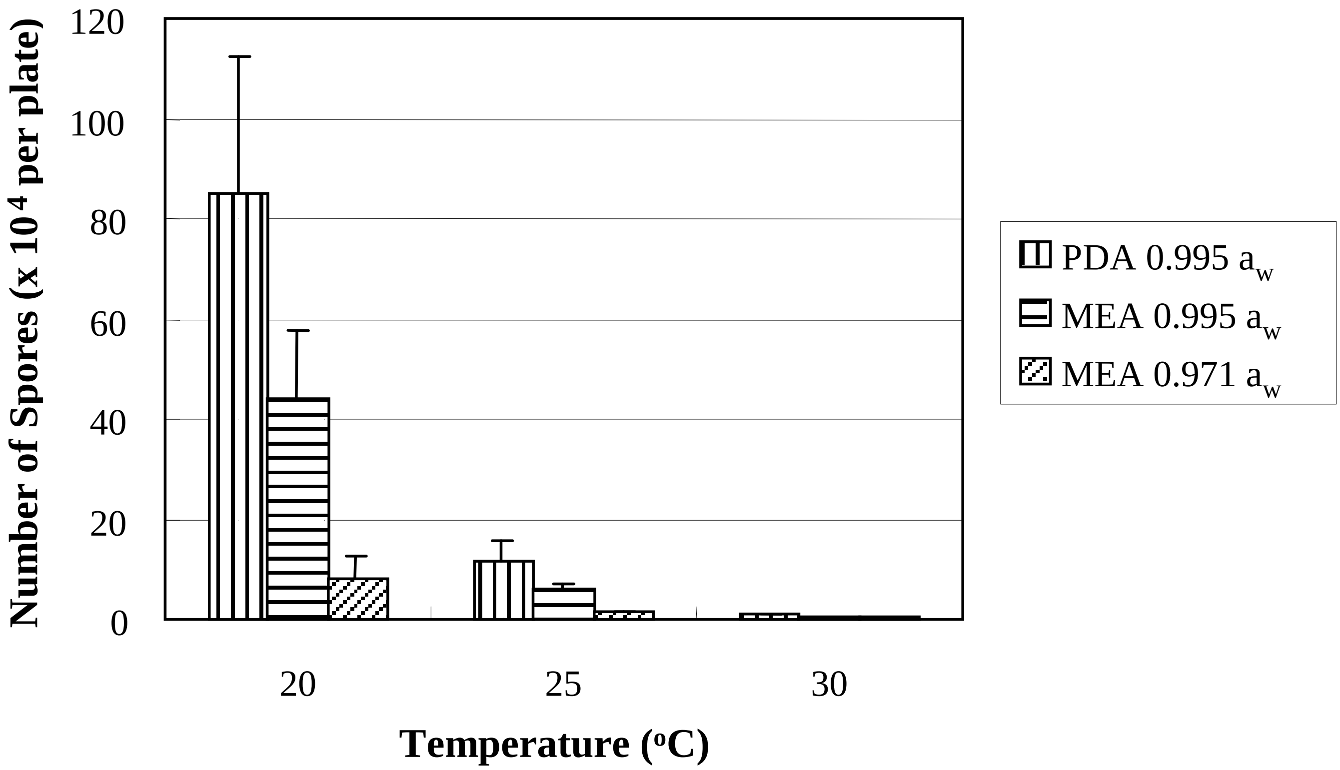

Figure 3. Lee and Magan

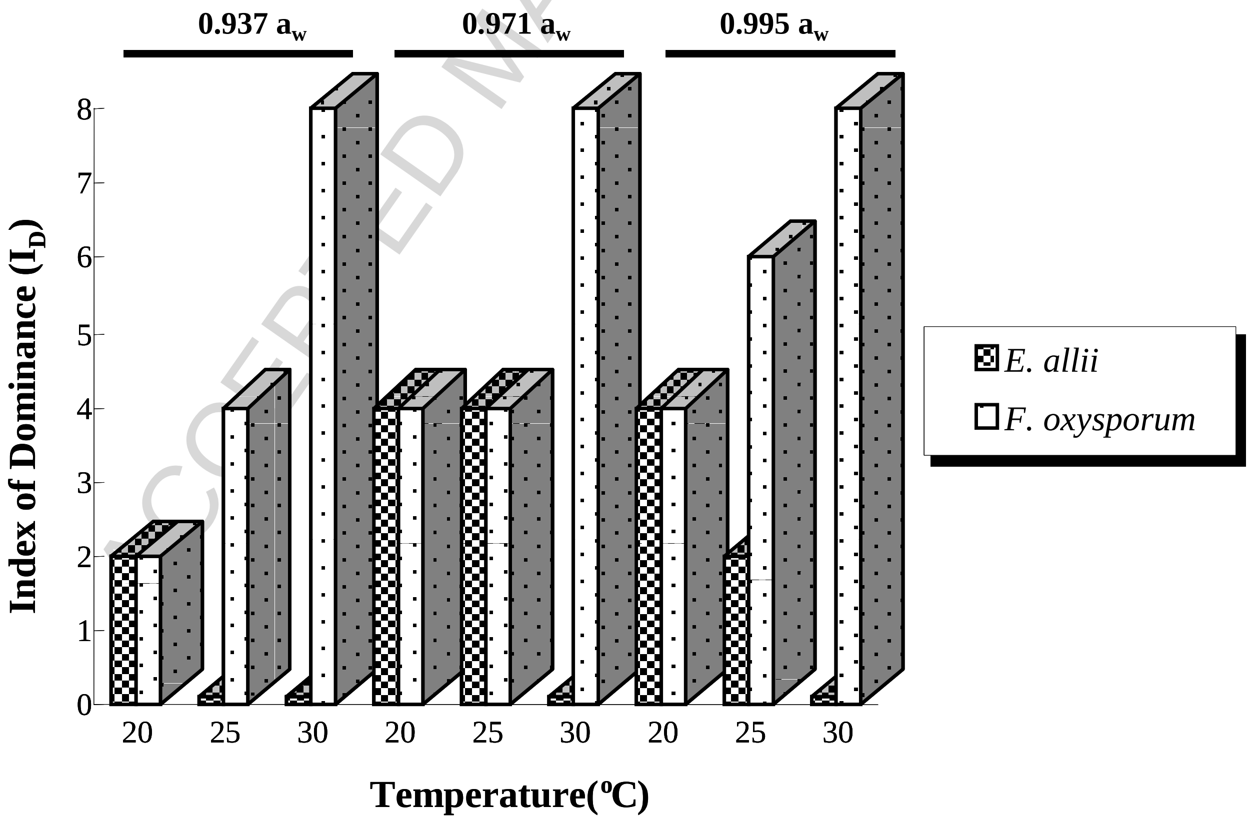

Figure 4. Lee and Magan

The influence of environmental factors on growth and interactions between Embellisia allii and Fusarium oxysporum f. sp. cepae isolated from garlic

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