Annals of Microbiology, **59 (4)** 663-674 (2009)

The conidia formation of several *Fusarium* species

Francisca SEMPERE*, María Pilar SANTAMARINA

Departamento de Ecosistemas Agroforestales, Escuela Técnica Superior del Medio Rural y Enología, Universidad Politécnica de Valencia, Avda. Blasco Ibañez 21, 46010 Valencia, España

Received 3 July 2009 / Accepted 12 October 2009

Abstract - Four strains of several species of the genus *Fusarium*: *F. culmorum*, *F. sambucinum* and *F. verticillioides* were investigated from the morphological and cultural point of view. To carry out the study two techniques were used: light microscopical and cryogenic scanning electron microscopical observations. The conidiogenous cells formation of all fungi took place inside of false heads from one conidiophore or more. The branched monophialides emerged from the degradation of false heads. Hypothetically *F. culmorum* and *F. sambucinum* no formed microconidia, what was observed that immature conidia in the substrate were consequence of the destruction of the false heads by a bad manipulation of the samples later of their processing. Sporulation of all strains was carried at the water activities of 0.995, 0.98 and 0.95 at both temperatures. The formation structure did not change with the temperature and the water activity of the strains but it was observed that increasing the value numeric of these abiotic factors their development was faster.

Key words: *Fusarium*; conidia formation; false heads; water activity; temperature.

INTRODUCTION

The geneneric concept of *Fusarium* was first diagnosed in 1809 by Link with the primary character being the presence of the distinctive canoe- or banana-shaped conidia well know to all researchers with eve a passing acquaintance with the genus (Leslie and Summerell, 2006).

The genus *Fusarium* contains over 66 species. Cultural characters for identification of species or this genus are principally shape and septa of macroconidia but presence or absence of microconidia, chlamydospores, circinate hyphae, production of crystals in the media, the ability of produce sclerotial-like structures, production of mesoconidia and the existence of sexual stage, are other characters could help to complement the characterization of the strains species.

Identification of *Fusarium* species is difficult due to great variation in morphological characteristics. Traditional studies of cultural characteristics in basic media, microscopic observation of the different reproductive structures and their conidia as well as the presence or absence of the other morphological structures have allow the identification and classification of the species until they appeared the molecular techniques. By means of these investigations a better classification has been possible but it is essential to complement with the classical techniques.

* Corresponding Author. Phone: +34 963 877414;
E-mail: frasemfe@yahoo.es

Actually many of the *Fusarium* species currently used in taxonomic systems are poorly defined and type specimens may not even exist. These descriptions probably could not be published today as they would not satisfy the requirements of the International Code of Botanical Nomenclature because the descriptions are vague or poorly defined, but they have been conserved due to priority and their wide usage (Leslie and Summerell, 2006).

In recent year equips of microscope observations have been evolutioned. The application of cryogenic scanning electron microscopy in previous researches carried in the laboratory of the Fungal Diversity Collection of Valencia Region have allowed the knowledge of new morphological aspects of the fungi not described previously (Sempere and Santamarina, 2008).

Fusarium culmorum (W.G Smith) Saccardo (sexual stage no known), *Fusarium sambucinum* Fükel *sensu stricto* (teleomorph *Gibberella publicaris* (Fries) Saccardo var. *publicaris*) and *Fusarium verticillioides* (Saccardo) Niremberg (teleomorph *Gibberella moniliformis* Wineland) are species commonly associated with cereals causing crop damages of different consideration.

The objectives of this study were (1) the comparison of the growth of the different strains at different temperatures and water activities (2) to examine the conidia's formation of *F. culmorum*, *F. sambucinum* and *F. verticillioides* by means of light microscopy and cryogenic scanning electron microscopy and (3) the study of sporulation at different temperatures and water activities.

MATERIALS AND METHODS

Microorganisms and culture medium. Strains of *Fusarium culmorum*, *Fusarium sambucinum* and *Fusarium verticillioides* were isolated at the laboratory of Agroforest Ecosystems of the School of Rural Environments and Enology from samples of rice grains collected from different rice fields and cooperatives of the main rice producing areas in Valencia. All fungal species were kept in Potato Dextrose Agar (PDA).

The synthetic medium used for the ecophysiological assay was Rice Extract Agar (REA) similar to rice composition with a pH of 5.5. To adjust the medium to the different water activities required, the substrate was modified by adding different amounts of glycerol (Sempere and Santamarina, 2006a).

Identification of fungal strains. The samples were identified at the laboratory of Fungal Diversity Collection of Valencia Region by means of classical and molecular techniques.

Fungi cultural characterization. Plates with the same water activities (a_w) were placed in water impermeable plastic containers together with two 100 ml beakers containing a glycerol water solution with an equilibrium relative humidity value identical to the a_w of the plates (Sempere and Santamarina, 2007). In this way, equilibration to the target a_w levels was achieved within 24 h, maintaining a constant relative humidity inside the Petri plates and also controlling the a_w of the substrate.

Disks of 9 mm diameter were cut out from actively growing pure cultures of the colonies of the isolates used in this study on PDA at 25 °C. The mycelial fragments were placed in the centre of 90 and 150 mm Petri plates containing REA. The samples were maintained during a period of 60 days.

In total 10 conditions were tested combining five water activities (0.85, 0.90, 0.95, 0.98 and 0.995) and two temperatures (15 and 25 °C).

The growth was measured after 5 days at intervals of 24 h according to the method described by Sempere *et al*. (2007). To calculate the growth rates (mm·day^{-1}) a linear regression of the radius (mm) as opposed to the time (days) was carried out. The computer software used was Microsoft Excel 2003.

Morphological study. After the cultural and morphological study in conventional media of the filamentous fungi they were examined in REA medium. Light Microscopy and Cryo-Scanning Electron Microscopy techniques were used to analyse the isolates of *F. culmorum*, *F. sambucinum* and *F. verticillioides* at different temperatures (15 and 25 °C) and water activities (0.95, 0.98 and 0.995).

For the light microscopy, dual microculture technique was carried (Sempere and Santamarina, 2006b, 2007). For the Cryo-SEM analysis of the samples, the species were inoculated at the same procedure of microculture technique but without coverslip.

To maintain the levels of water activity, filter paper disks impregnated with different water levels (0.995, 0.98 and 0.95 a_w: 2.5, 11 and 23.5 g glycerol/100 mL distilled water) were aseptically placed on the Petri plates.

After the incubation period, the squares of REA medium were mounted on a stub and were frozen in liquid nitrogen. Its were subsequently sublimed at -90 °C for 15 min, covered with gold for 30 s, and visualized by scanning microscope at 10 kV. The type of signal detected for this microscope was secondary electrons.

Microscopic examination of samples grown alone was performed 7-30 days after inoculation depending on the water activity and temperature assayed.

RESULTS

Ecophysiological study

Maximum growth rate of *F. verticillioides* grown alone occurred at a temperature of 25 °C and 0.995 a_w. The growth colony was of 5.16 mm day^{-1} (Fig. 1).

The minimum amount of water activity producing growth in REA occurred at 0.90 a_w. Although initially development was not registered at 0.95 a_w and 15 °C, and at 0.90 a_w at both temperatures, it was observed during the eight testing weeks.

Fusarium culmorum registered the maximum growth of the three strains followed by *F. sambucinum* and *F. verticillioides* in all conditions assayed (Tables 1 and 2). Only the numeric value registered for growth of *F. sambucinum* was bigger than the value of *F. culmorum* at 0.90 a_w at 25 °C. At the same conditions but with other substrate – rice - the development of the species was the same in order, but with different growth rates (data not shown).

Similar growth rates of *F. verticillioides* at 0.98, 0.995 aw at 15 °C and 0.95 aw at 25 °C were observed (Fig. 1).

Single factors (water activity, temperature) had a significant effect on fungal growth of *F. verticillioides* ($P < 0.01$) (Table 3).

Cultural characterization

Fusarium culmorum formed filamentous colonies of different reddish-orange shades at the temperatures and water activities assayed. Whitish-pink to orange cotton-like aerial mycelium, more abundant at high water activities -0.98 and 0.995 a_w. The development of the tested strains at 0.95 a_w was more irregular with the margin lobulated. Occasionally, this strain generated a whitish exudation. Reverse of the strains were reddish to pink (Fig. 2).

Unlike of *F. culmorum*, *F. sambucinum* showed orange colonies. At 0.95 a_w and 25 °C, when the colony matured, it was transformed the orange to brownish-blackish colour. Meanwhile at the same water activity at 15 °C and at both temperatures at 0.98, the shade of the colonies were increased in their coloration. Whitish cotton-like aerial mycelium more abundant at 0.98 a_w and both temperatures. Reverse of the colonies orange to brown. Occasionally, the mature colonies generated a whitish exudation (Fig. 3).

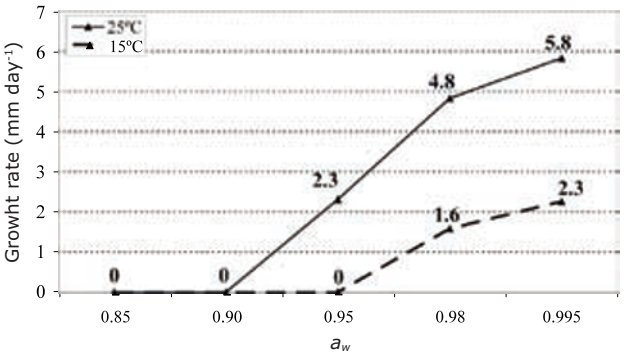

FIG. 1 - Temperature-dependent influence of water activity on the growth rate of *Fusarium verticillioides* grown individually in Rice Extract Agar.

TABLE 1 - Growth rates of *Fusarium culmorum*, *Fusarium sambucinum* and *Fusarium verticillioides* grown alone at 25 °C and different water activities

Water activity	*Fusarium culmorum*	*Fusarium sambucinum*	*Fusarium verticillioides*
0.85	0	0	0
0.90	0.15	0.45	0
0.95	2.7	2.41	2.3
0.98	6.48	5.41	4.8
0.995	9.76	6.7	5.8

TABLE 2 - Growth rates of *Fusarium culmorum*, *Fusarium sambucinum* and *Fusarium verticillioides* grown alone at 15 °C and different water activities

Water activity	*Fusarium culmorum*	*Fusarium sambucinum*	*Fusarium verticillioides*
0.85	0	0	0
0.90	0	0	0
0.95	1.48	1.41	0
0.98	3.98	2.88	1.6
0.995	6	3.13	2.3

TABLE 3 - Analysis of variance of the growth rate of *Fusarium verticillioides*; significance of water activity (a_w), temperature (T) and their interaction (a_w x T)

Factor	DF	MS	F-ratio	P-value
a_w	4	793.392	51.99	0.0000**
T	1	1618.81	106.07	0.0000**
a_w x T	4	333.833	21.87	0.0000**

DF: Degrees of freedom. MS: Mean squares. **Indicates that the factor elicited a significant effect ($P < 0.01$).

Scarce and whitish aerial mycelium and colonies of different pink shades were the macroscopic characters observed when the colonies were analysed *F. verticillioides* at the different temperatures and water activities (Fig. 4).

Morphological characterization and formation of conidia of *Fusarium* species

Sporulation of all strains of the three species *F. culmorum*, *F. sambucinum* and *F. verticillioides* was observed at all water activities and temperatures assayed (Fig. 5, 6, 7, 8, 9, 10, 11). Morphological analysis of filamentous fungi was discarded in this study for water activities of 0.85 and 0.90 since the development of the strains was null or minimal after testing period.

The monophialides' formation of *F. culmorum* was produced inside of the globose or subglobose false heads with a size of 6.9 µm (Fig. 6A and 6B). The false heads development took place from one conidiophore (Fig. 5A, 5B, 5C and 5D) or two (Fig. 6E, 6F (see the indicator) and 6G).

At first the false heads often appeared coiled around of the hypha of the conidiophore and they were unrolled at the same time that they increased their size until all structure was straight (Fig. 6A, 6B, 6C, 6E and 6G). Finally the false heads' wrappers broke down by the pressure exerted by the monophialides and the conidia with different degree of development (Fig. 5C, 5E, 5F and 6H).

Conidia's apical cells of *F. culmorum* usually are rounded but in some isolates it is slightly papillate which can lead to confusion with those of *F. sambucium*. The four isolates of *F. culmorum* showed an apical cell slightly papillate but the upper growth rate of these strains confirmed with other analysis that the isolates were *F. culmorum*. In the studied samples usually 3 or 4 numbers of septa were shown by conidia, occasionally 5. The strains formed chlamydospores smooth-walled, singly, in clumps or chains. The microscope observation of *F. culmorum* showed seldom the formation of conidiophore into the false heads.

The formation of conidiophores of *Fusarium sambucinum* was carried at the same way of *F. culmorum* (Fig. 7 and 8). In this sense, the size of false heads was bigger and their shape was sometimes ellipsoidal (Fig. 7B, 7D, 8C, 8D, 9E and 9F) .The upper size of *F. sambucinum* false heads was consequence of the intervention of more conidiophores (Fig. 7D and 9E).The conidiophores arising as single, later branching sparsely, and verticillately branched.

Other morphological character that differenced both species was that the conidia of *F. culmorum* were wider that *F. sambucinum* (Fig. 9C and 9D). The different strains of this latter species formed smooth macroconidium with 3-5 transversal septa with apical cells papillates and basal cells foot-shaped. These filamentous fungi formed smooth chlamydospores in chains and cluster in hyphae either on the surface or submerged in the agar (Fig. 9A and 9B).

Fusarium culmorum and *F. sambucinum* did not formed microconidia. Hypothetically the observation of inmature macroconidia was consequence of the destruction of false head by a bad manipulation of the samples.

In the aerial mycelium of the strains of *Fusarium verticilliodes* was observed long chains of microconidia emerging of aggregates microconidia and false heads (Fig. 10H, 11D and 11E). Sometimes it was difficult to distinguish if the conidia were formed in false heads or in aggregates microconidia (Fig. 10C, 10D, 10E, 10F and 10G 11A, 11B and 11C). The conidiogenus cells were formed in branched or unbranched monophialides (Fig. 10C, 11A, 11B and 11C). An abundant formation of thin-walled oval to club shaped usually no septate microconidia also emerged of monophialides.

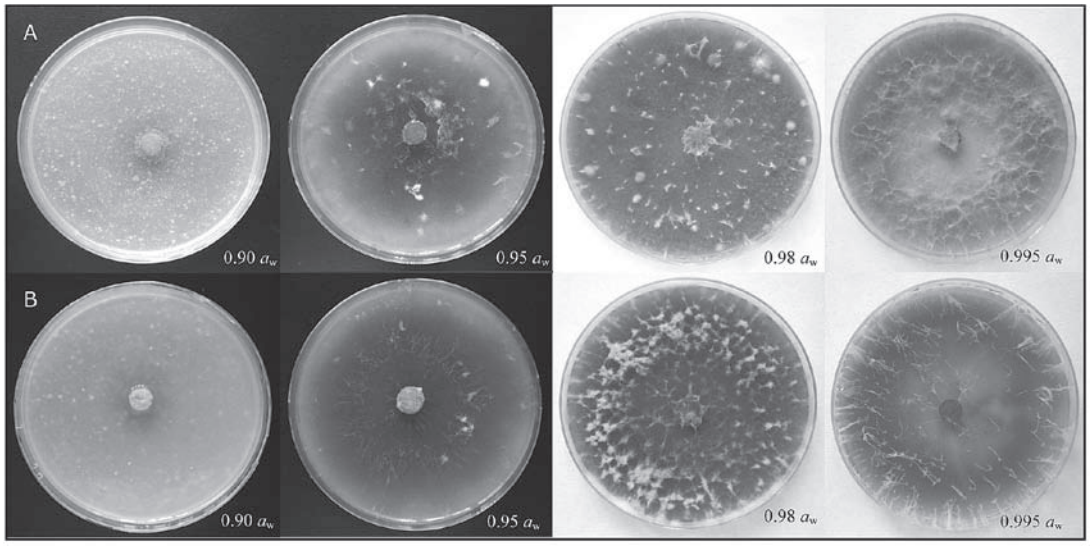

FIG. 2 - *Fusarium culmorum´*s colonies after 8 weeks of growth at 25 ºC and at different water activities. Row A: 25 ºC. Row B: 15 ºC.

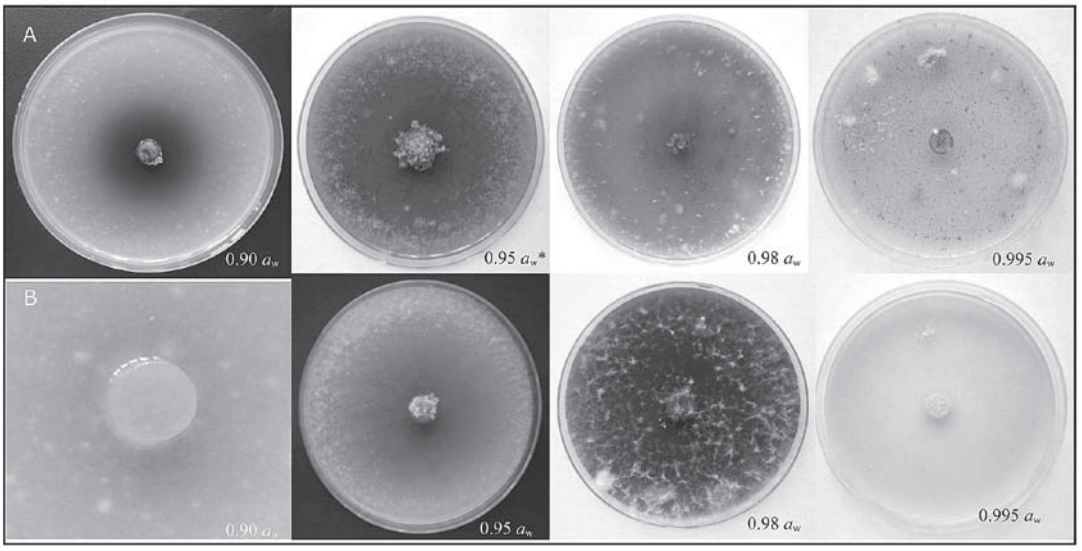

FIG. 3 - *Fusarium sambucinum´*s colonies after 8 weeks of growth at 25 °C and at different water activities. Row A: 25 °C. Row B: 15 °C. * 4 weeks of growth.

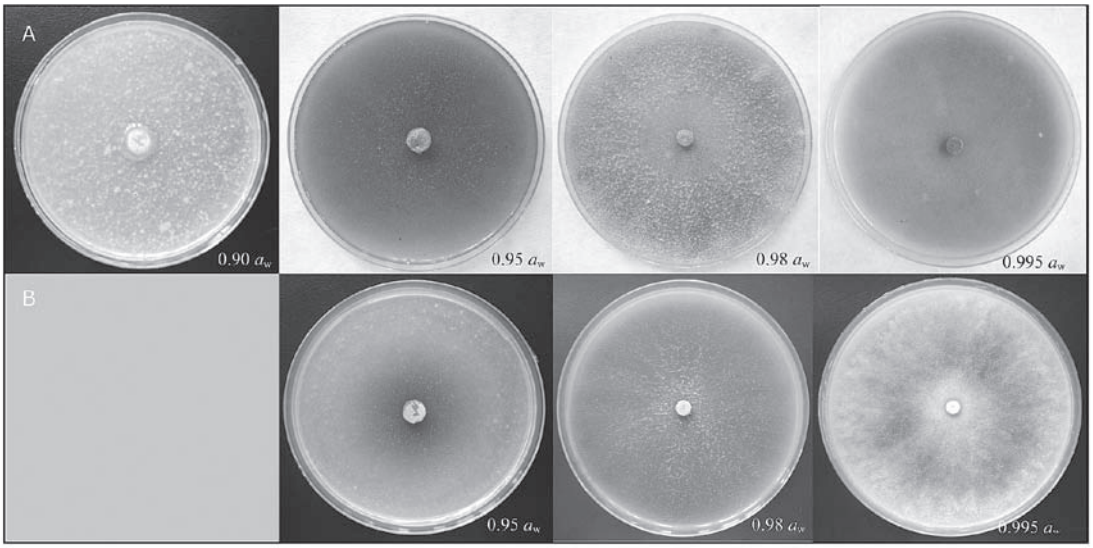

FIG. 4 - *Fusarium verticillioides´* colonies after 8 weeks of growth at 25 °C and at different water activities. Row A: 25 °C. Row B: 15 °C.

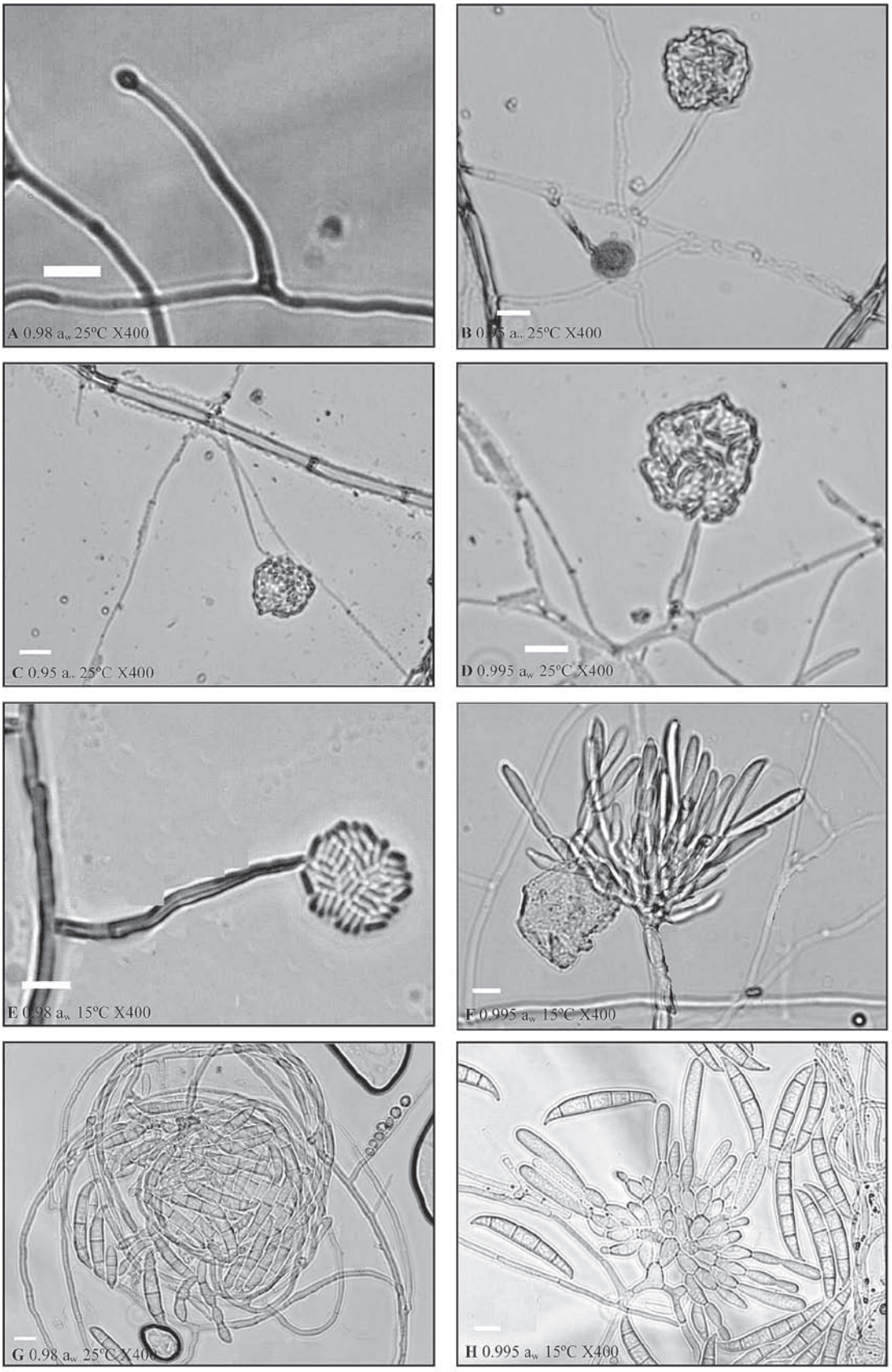

FIG. 5 - Light micrographs showing the conidia´s formation of *Fusarium culmorum* at different temperatures and water activities. A, B, C: Different degree of formation of conidial false heads. D: Initial rupture of the false head. E: Conidia disposition into the false head. F: Detachment of the false head´s wall. G: Disappearance of false head and unrollement of conidiophore H: Conidiophore completely unrolled, where it can be seen some mature macroconidia are scattered in the substrate. X 400. White bars: ≈5 µm.

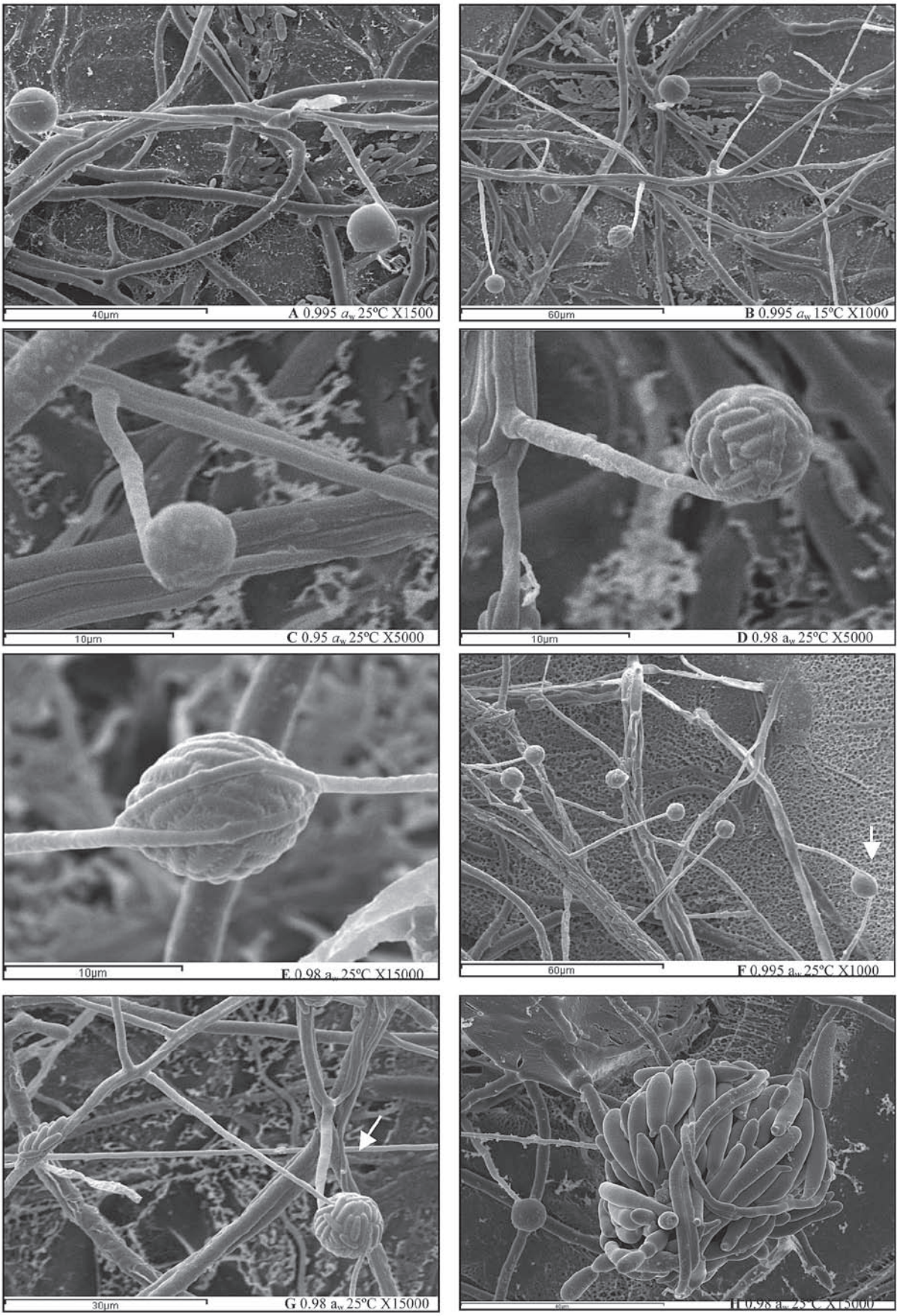

FIG. 6 - Cryo-scanning electron micrographs showing the conidia´s formation of *Fusarium culmorum* at different temperatures and water activities. A, B,C, D: Initial formation and different development degree of *F. culmorum* conidiophores from the false heads. In these photographs the false heads have been formed from a conidiophore. E, F, G: Formation of false head from two conidiophores (see the arrow). G: Straight conidiophore with the conidia still immature. H: Conidiophore completely formed.

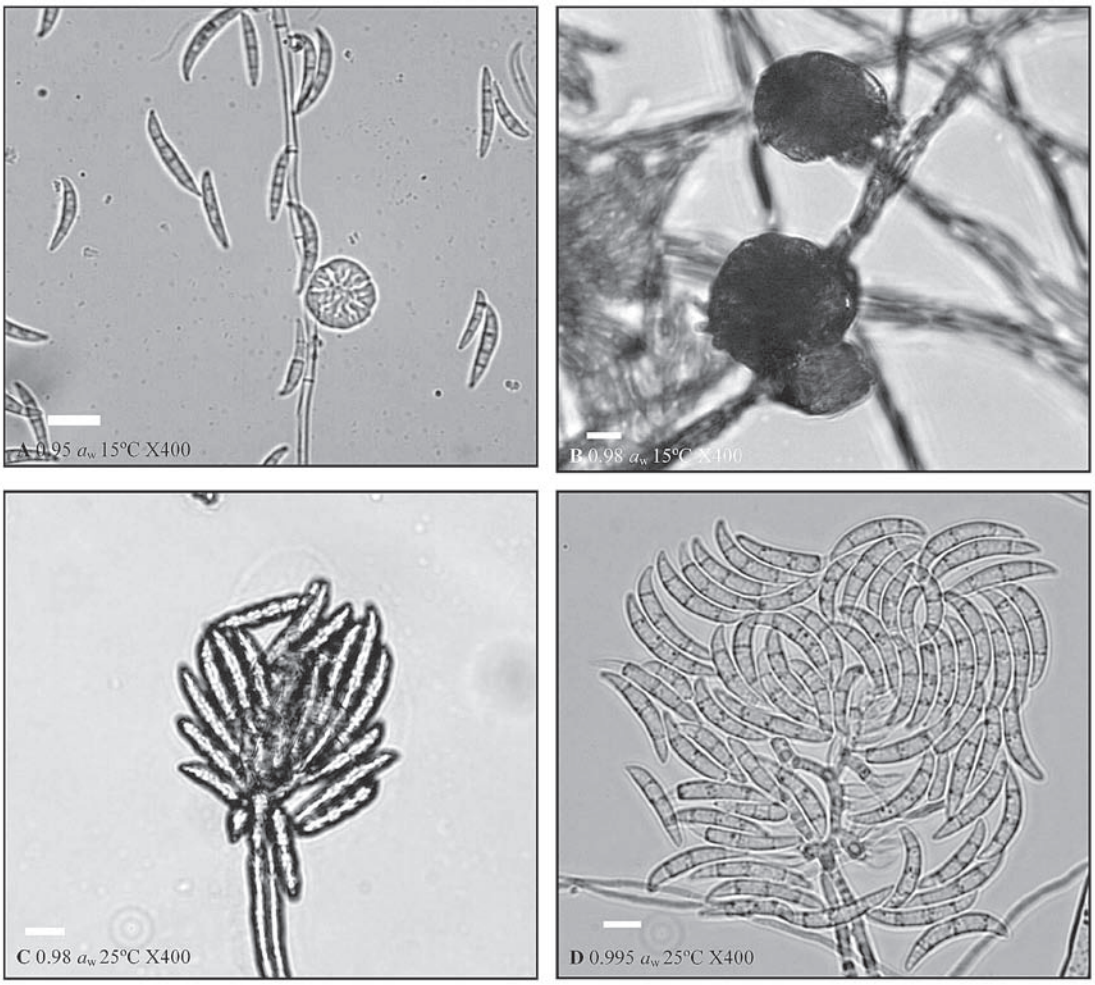

FIG. 7 - Light micrographs showing the conidia's formation of *Fusarium sambucinum* at different temperatures and water activities. A, B: False heads at different development degrees. C: It can be seen the wall's structure of the degraded false head where it's emerge the macroconidia. D: Conidiophores without false head where it can be seen the conidia at different development degrees. White bars: ≈5 µm.

Fusarium verticillioides in Rice Extract Agar formed long and slender scarce macroconidia with a curved apical cell and often tapered to a point with a basal cell notched or foot shaped with 3 or 5 septa. The strains did not form chlamydospores.

DISCUSSION

Formation of false heads with different characteristics in the genus *Fusarium* has been described for over 41 species. The presence of false heads in *F. sambucinum* and *F. verticillioides* has been previously reported but have not been described their formation. Anyway this is the primer research with report the formation the false heads by *F. culmorum*.

Previous studies have detailed the important influence of abiotic factors such as a_w, temperature on the ability of *Fusarium* species to germinate, grow and produce mycotoxins (Miedaner and Perkowski, 1996; Picco *et al*., 1999; Hope and Magan, 2003; Llorens *et al*., 2004; Sempere *et al*., 2004, 2007). Previously minimal values for growth of *F. verticillioides* of 0.87 and 0.89-0.90 a_w have been described (Woods and Duniway, 1986; Marín *et al*., 1995, 1998; Pitt and Hocking, 1999).

Just like that *F. verticillioides*, Sempere *et al*. (2004, 2007) reported the same maximum growth at 0.995 a_w and 25 °C for the other rice *Fusarium* sp., *Fusarium culmorum* and *F. sambucinum*, grown individually and jointly others strains. Velluti *et al*. (2000) obtained the highest growth rate for *F. verticillioides* at 0.98 aw and 25 °C on maize but this water activity was the maximum value experimented.

Fusarium culmorum may be confused with *F. sambucinum* or *F. crookwellense*, as all can be isolated from similar hosts and climatic regions (Leslie and Summerell, 2006).

According Wagacha and Muthomi (2007) cereal fusaria do not form microconidia, except under certain cultural conditions, and are generally distinguished on the basis of morphology of the macroconidia. The formation of microconidia has been reported for *F. sambucinum* by Leslie and Summerell (2006) but no for Samson *et al*. (2004). These researchers and others not reported microconidia formation for *F. culmorum*. On the other hand all researchers coincide with the formation of conidia of *F. verticillioides*. This investigation reported the formation of microconidia only for *F. verticillioides*.

In this study it was observed that the rupture of the false heads was carried by the pressure exerted by the growth of the

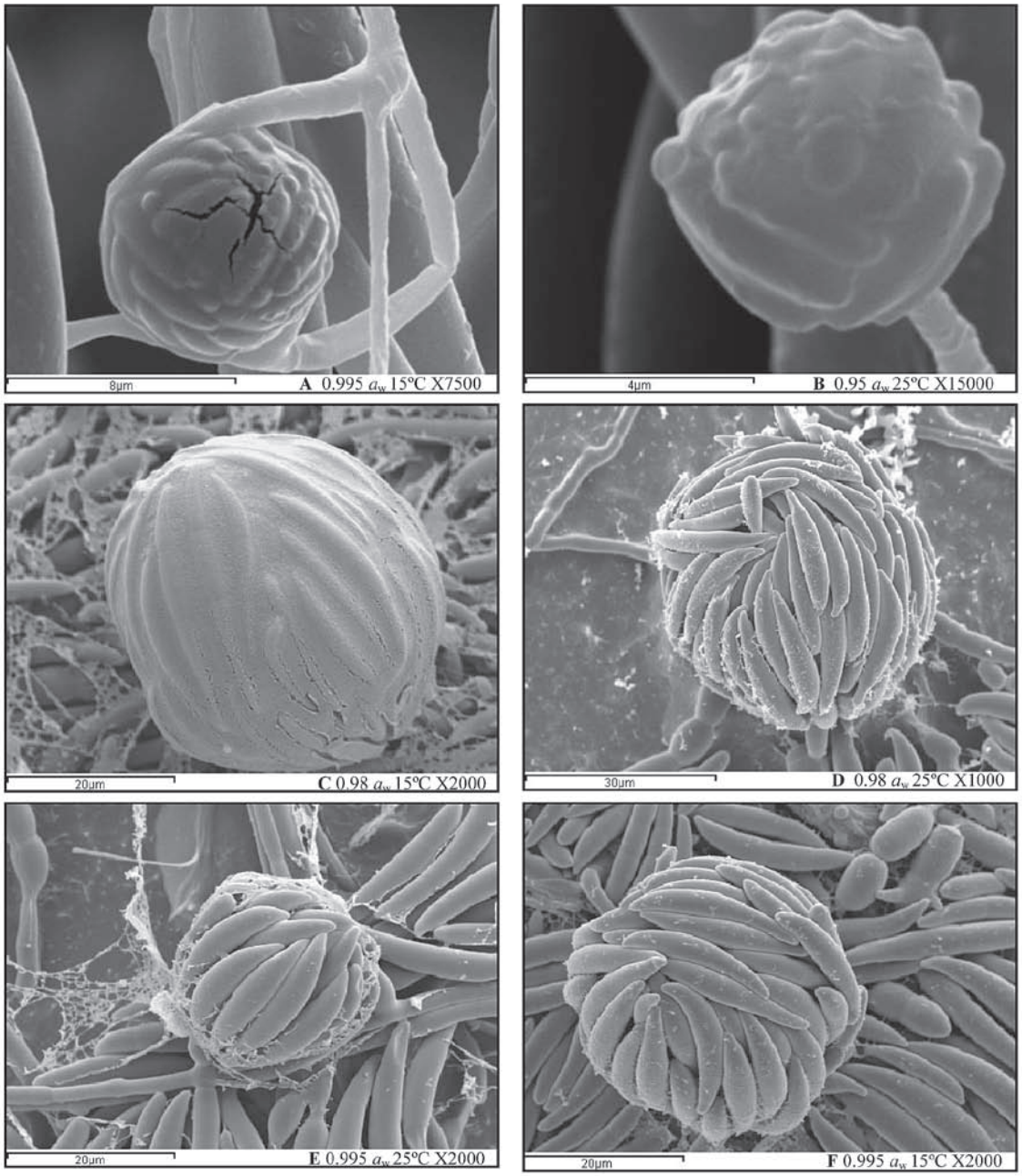

FIG. 8 - Cryo-scanning electron micrographs showing the conidia´s formation of *Fusarium sambucinum* at different temperatures and water activities. A, B, C, D: False heads at different development degrees. E: It can be seen the wall´s structure of the degraded false head where it´s emerge the macroconidia. F: Conidiophore without false head.

conidia and phialides. However others factors may be involved: fungi metabolites, etc. In addition it was suggested that the number of conidiophores in the formation of the false heads a differential character between species.

The application of this technical allow to find characters of Fungi Kingdom so far not described for other researchers and it is less tedious that other techniques employed actually (Sempere and Santamarina, 2007; 2008). The technical's problem is what the sample has to handle with precaution and low voltage.

Nevertheless, more assays over the genus *Fusarium* are necessary for a right classification and characterization of its species.

REFERENCES

Hope R., Magan N. (2003). Two-dimensional environmental profiles of growth, deoxynivalenol and nivalenol production by *Fusarium culmorum* on a wheat-based substrate. Lett. Appl. Microbiol., 37: 70-74.

Leslie J.F., Summerell B.A. (2006). The Fusarium Laboratory Manual. Blackwell Publishing.

Llorens A., Mateo R., Hinojo M.J., Valle-Algarra F.M., Jiménez M. (2004). Influence of environmental factors on the biosynthesis of type B trichothecenes by isolates of *Fusarium* spp. from Spanish crops. Int. J. Food Microbiol., 94: 43-54.

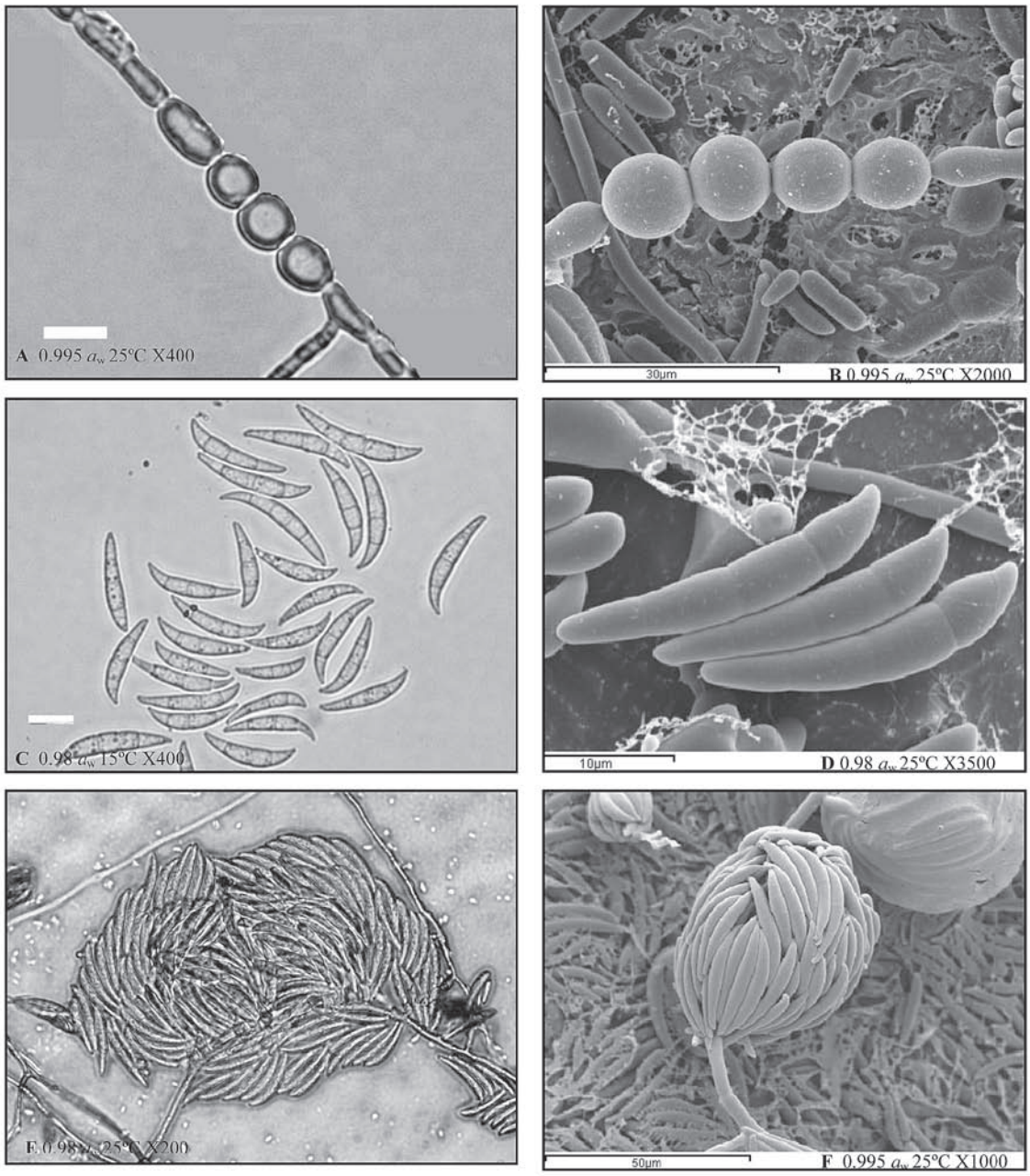

FIG. 9 - Light and cryo-scanning electron micrographs of *Fusarium sambucinum*. A, B: Chlamidospores. C, D: macroconidia. E, F: Disposition ellipsoidal of the different conidiophores without false head. White bars: ≈5 µm.

Marín S., Sanchis V., Magan N. (1995). Water activity, temperature and pH effects on growth of *Fusarium moniliforme* and *F. proliferatum* isolates from maize. Can. J. Microbiol., 41: 1063-1070.

Marín, S., Sanchis, V., Rull, F., Ramos, A.J., Magan N. (1998). Colonization of maize grain by *Fusarium moniliforme* and *Fusarium proliferatum* in the presence of competing fungi and their impact on fumonisin production. J. Food Protect., 61: 1489-1496.

Miedaner T., Perkowski J. (1996). Correlations among *Fusarium culmorum* head blight resistance, fungal colonization and mycotoxin contents in winter rye. Plant Breeding, 115: 347-351.

Picco M., Nesci A., Barros G., Cavaglieri L., Etcheverry M. (1999). Aflatoxin B1 and fumosin B1 in mixed cultures of *Aspergillus flavus* and *Fusarium proliferatum* on maize. Natural Toxins, 7: 331-336.

Pitt J.I., Hocking A.D. (1999). Fungi and Food Spoilage, 2nd edn., Aspen Publishers, Inc., New York.

Samson R.A., Hoekstra E.S., Frisvad J.C. (2004). Introduction to Food- and Air-Borne Fungi, 7th edn., Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Sempere F., Santamarina M.P. (2006a). Ecofisiología de *Drechslera oryzae* Subram. & Jain en condiciones *in vitro*. Phytoma, 178: 49-50.

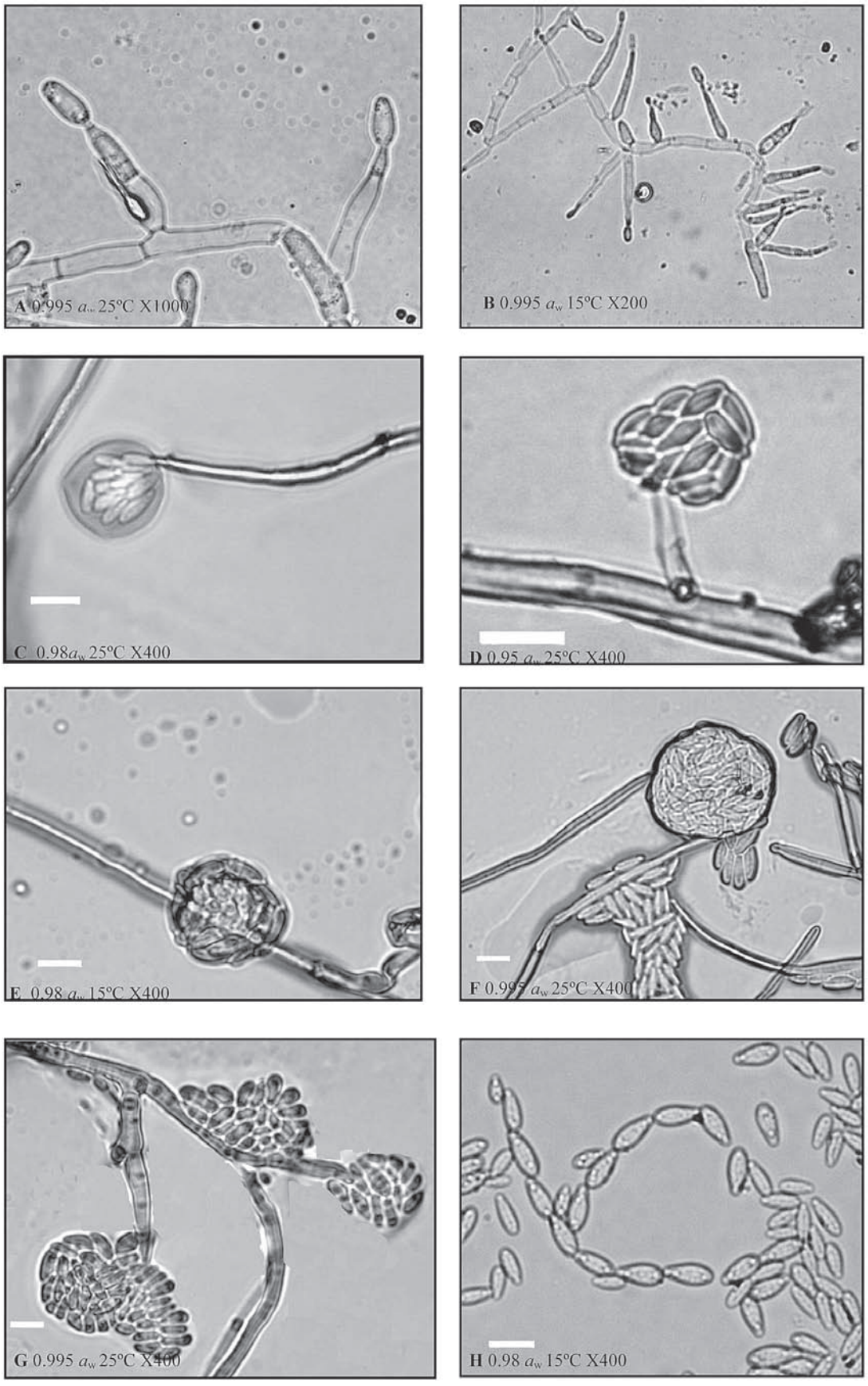

FIG. 10 - Light micrographs showing the conidia´s formation of *Fusarium verticillioides* at different temperatures and water activities. A, B: Initial formation of conidiophore. C: It can be seen the false head. D, E, F, G: Different development degree of false heads´ formation. H: Long chains of macroconidia. White bars: ≈ 5 µm.

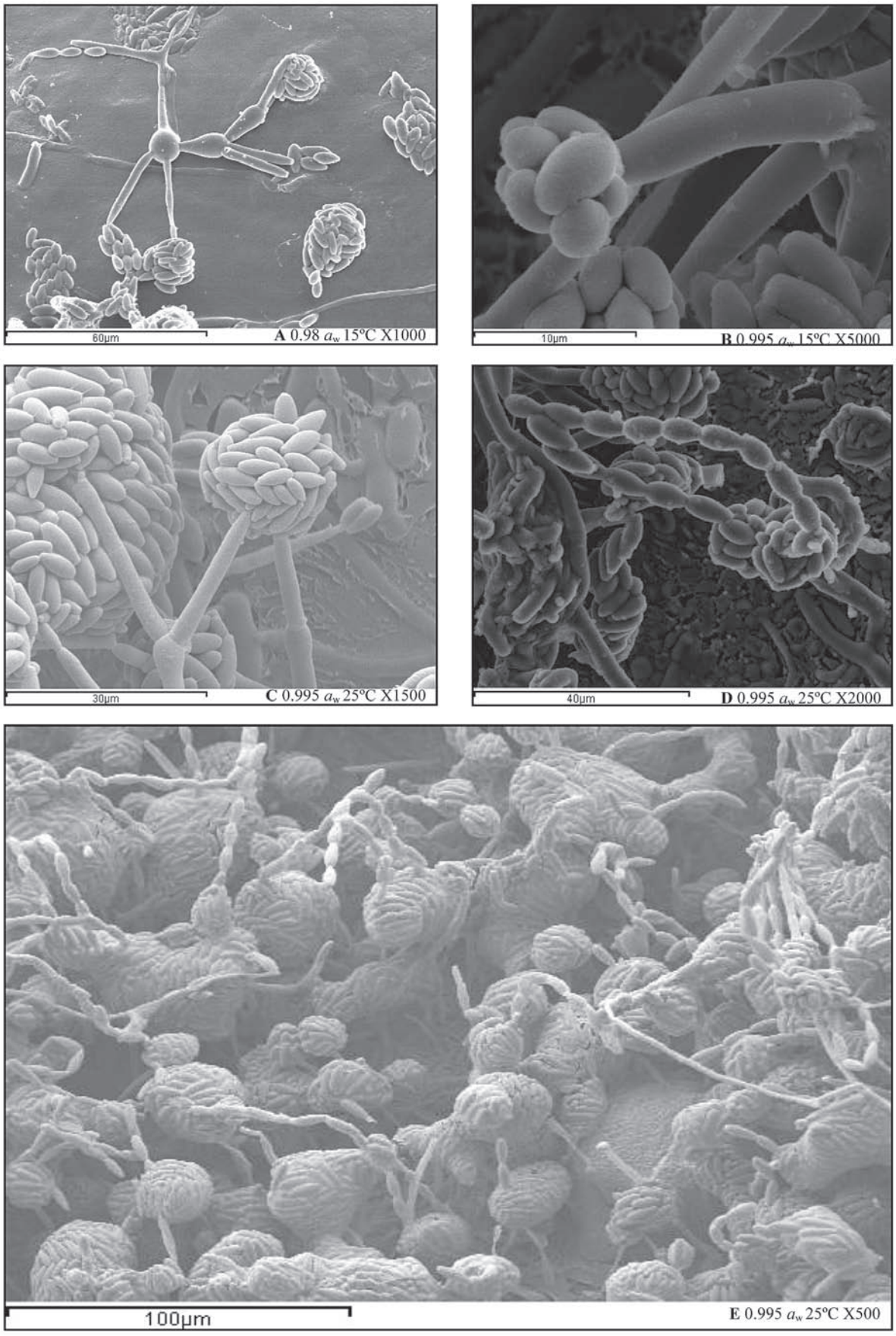

FIG. 11 - Cryo-scanning electron micrographs showing the conidia´s formation of *Fusarium verticillioide*s at different temperatures and water activities. A, B, C: Initial formation and different development degrees of false heads. D: Long chains of microconidia emerging of two false heads. E: General prospect of development of *F. verticillioides*.

Sempere F., Santamarina M.P. (2006b). Microscopic and macroscopic study of the interaction between *Alternaria alternata* (Fr.) Keissler and *Nigrospora oryzae* (Berk. & Broome) Petch. Ann. Microbiol., 56: 101-107.

Sempere F., Santamarina M.P. (2007). *In vitro* biocontrol analysis of *Alternaria alternata* (Fr.) Keissler under different environmental conditions. Mycopathologia, 163: 183-190.

Sempere F., Santamarina M.P. (2008). Suppression of *Nigrospora oryzae* (Berk. & Broome) Petch by an aggressive mycoparasite and competitor, *Penicillium oxalicum* Currie & Thom. Int. J. Food Microbiol., 122: 35–43.

Sempere F., Roselló P., Santamarina M.P. (2004) Estudio ecofisiológico de *Fusarium culmorum* (G.W. Smith) Sacc. en condiciones *in vitro*. Phytoma, 162: 49-50.

Sempere F., Roselló J., Santamarina M.P. (2007). Interacciones competitivas entre *Fusarium sambucinum* Fuckel y *Phoma glomerata* (Corda) Wollenweber & Hochapfel en condiciones *in vitro*. Rev. Iberoam. Micol., 24: 29-33.

Velluti A., Marín S., Bettucci L., Ramos A.J., Sanchis V. (2000). The effect of fungal competition on colonization of maize grain by *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum* and on fumonisin B and zearalenone formation. Int. J. Food Microbiol., 59: 59-66.

Wagacha J.W., Muthomi J.W. (2007). *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. Crop Prot., 26: 877-885.

Woods D.M., Duniway J.M. (1986). Some effect of water potential on growth, turgor, and respiration of *Phytophtora cryptogea* and *Fusarium moniliforme*. Phytopatology, 76 (11): 1248-1254.