The effect of some environmental conditions on the growth and activity of the external enzymes for five sp. of *Fusarium*

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Abstract
A Laboratory studies are conducted to determine the influence of environmental parameters (temperature, pH, light regime and type of growth medium) on growth of five species of *Fusarium* fungi. The highest growth *of F.oxysporum and F.solani* is obtained at 25˚C and gives growth (8 and 7.5 cm respectively) after 6 days of incubation. In contrast *Fusarium proliferatum* reveals maximal growth (8 cm) at 30˚Cduring the same period while, the *F.sacchari and F.globosum* grow well at 20˚C. The optimum pH for growth of all species of *Fusarium* is obtained at 7.5. *F.oxysporum and F.solani* reveals best growth on SDA. Whereas, *F.globosum, F.proliferatum, and F. sacchari* are showed higher growth on CDA. The growth of all studied fungi is reduced significantly under continuous light compared with that grow at dark period. The ability of *Fusarium* species for producing extracellular enzymes (protease and cellulose) is examined by using CMC and skim milk. Among all tested species, *F.oxysporum and F.sacchari* was showed high protease activity at the third day of incubation, compared with *F.globosum, F. proliferatum and F. solani* which reveals less activity for protease. The experiment suggested strong chitinase activity *F.oxysporum and F.sacchari*, while other species of *Fusarium* showed least activity. Furthermore, cellulose activity for all species of *Fusarium* are detected.
Kewords: Fusarium species; environmental conditions; mycelial growth; extracellular enzymes

الخلاصة
اجريت دراسات مختبرية لتحديد تأثير التغيرات البيئية مثل الحرارة، pH والنظام الضوئي، ونوع الوسط الغذائي على نمو خمسة انواع من الفطر فيوزاريم. حيث لوحظ النمو المثالي للفطر*F.oxysporum* و*F.solani.* عند 25˚م واعطى مستعمرة فطرية بقطر 8 و7.5 سم على التوالي في اليوم السادس من الحضانة. في المقابل اعطى فطر *Fusarium proliferitum* اقصى معدل للنمو وقطر مستعمرة فطرية 8 سم عند درجة حرارة 30˚م في اليوم السادس من الحضانة. في المقابل قد شوهد النمو المثالي للفطرين *F.sacchari,* *F.globosum* عند درجة حرارة 20˚م. ظهر أفضل نمو لجميع انواع الفيوزاريم المدرسة عند مستوى الحموضة 7.5. ازداد نمو *F.oxysporum* و *F. solani* نموا معنويا على وسط السابرويد مقارنة ببقية الاوساط الغذائية ، بينما اعطت الفطريات *F.globosum,* *F.proliferatum, F. sacchari* اقصى نمو على وسط الزابك. كان نمو أنواع الفيوزاريم في الظلام اعلى معنويا من نموها تحت الضوء. تم تقييم انواع الفيوزاريم على انتاج الانزيمات خارج خلوية باستخدام اوساط غذائية حاوية على المادة الاساس مثل (الكايتينن ، كار بوكسي مثيل سيليلوز و حليب الفرز). اذ أظهر الفطرين (*F.oxysporum* و*F.sacchari*) فعالية عالية لإنزيم البروتيز في اليوم الثالث من الحضانة بالمقارنة مع *F.globosum, F. proliferatum and F. solani* التي اعطت فعالية اقل للبروتيز. بينت التجربة وجود فعالية عالية لإنزيم الكايتنيز للفطر*F.oxysporum* و *F. sacchari* مقارنة بمثيلاتها من الانواع الاخرى. تم الكشف ايضا عن انزيم السليلوليز في انواع الفيوزاريم.
الكلمات المفتاحية: انواع الفيوزاريم; الظروف البيئية;نمو المايسليم; الانزيمات خارج خلوية

1- Introduction
Fusarium is first discovered by link 1809 *Fusarium* has a worldwide distribution and considered to be the most important plant disease pathogens such as crown rots, head blights, scabs, vascular wilts, root rots and cankers (Leslie and Summerell ,2006), and seed contaminate that producing mycotoxins (Miller, 2001). Others, cause diseases in humans and animals particularly in neutropenic, transplant patients and are thus hazardous to agricultural products, wild life, livestock and humans (Austen *et al*., 2001).

In addition to diversity and distribution around the world, poisonous substances produced by *Fusarium* species (Logrieco *et al*. 2002), *Fusarium* species are causative agents of superficial and systemic infections in humans (Mayayo *et al*., 1999). Trichothecenes, zearalenone, and fumonisins, for instance, are the main *Fusarium* mycotoxins. *F. verticillioides*, *F. proliferatum*, and *F. nygamai* produced mycotoxins called fumonisins (Sorensen, 2009).

Reproductive structures are very important for identification of *Fusarium* species. They produce three types of spores; macro conidia, micro conidia, and chlamydospors (De Hoog *et al*., 2000).

Growth of *Fusarium* species are affected by different ecological conditions such as temperature, pH, light, and culture media etc. The optimal growth of *F. oxysporum* is found between 25-28 ^{0}C, inhibited above 33 ^{0}C and retarded below 17 ^{0}C (Cook and Baker, 1983). The growth of this fungus is reduced rapidly at pH 4, compared with that at pH 6 or 7 (Gruenwald *et al*., 2006). Composition of culture media also affecte the growth of *Fusarium* species, in addition to temperature and pH. It is reported that continuous light is more suitable for fungal growth than continuous darkness (Alam *et al*., 2001). *Fusarium* species release extra cellular enzymes which break down the pectin of the cell wall of many plants. These enzymes are applied in the industrialization of detergent, starch, drinks, food, textile, animal feed, chemicals and bio medical products. Among these enzymes, protease, cellulose, chitinase, lipase, and pectinase are found (Dias *et al*., 2010; Sumantha *et al*., 2006). Proteases are complex enzymes that differ from each other in properties such as substrate specifity, active site and mechanisms of action (Rao *et al*., 1998). Chitinase is a polymer of unbranched chains of β- 1,4 linked 2- acetamido- 2- deoxy – D- glucose, as fungal cell wall is rich in chitin (Peberdy, 1990). Cellulose is most abundant component of plant biomass. It is found in nature exclusively in plant cell wall. Any process which could efficiently and economically convert cellulytic material to glucose would be of immense industrial significance (Walsh, 2002). Fungi are the major agents of cellulose degradation (Lederberg, 1992). The aim of the current work is to study the role of different pH, temperature, growth media in addition to light and darkness on growth pathogens and their activities for produces enzymes.

2- Materials and Methods

2-1 Growth Media

a- Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA) and Sabouraud Dextrose Agar (SDA) are prepared according to Indian Production Company HEMIDIA.

b- Milk agar Media: skimmed milk powder is dissolved in 10 ml of distilled water and stir until it is completely dissolved. Dissolving 2 gram from agar in 90 ml of distilled water and adjusting the pH to 6. Both solutions were sterilized separately and mixed after cooling to 45 ^{0}C. This media is poured in sterile petri dishes (Bilinsk, 1987).

c- Carboxy methyl cellulose agar medium: CMC agar medium consists of 10gm Carboxy Methyl Cellulose (CMC), 2gm Sodium Nitrate ($NaNO_3$), 1gm Potassium Dihydrogen Phosphate (K_2HPO_4), 0.5gm Potassium Chloride (KCl), 0.5gm

Magnesium Sulphate ($MgSO_4.7H_2O$) and 20 gm Agar-Agar. This medium is prepared by dissolving all of material mentioned above in distil water except the (CMC) that gradually added by using magnetic stirrer with heating to mix the medium and adjust pH to 6. Media is sterilized in autoclave at 121 ^{0}C for 15 minutes, and distributed after sterilizing in sterile glass tubes at range 20-25 ml for each tube and waited to solidify.

2- Source of *Fusarium* isolates

The isolates of *Fusarium* are obtained from the Unit of Advanced Mycology / Department of Biology / College of Science/ University of Babylon / Autum 2013.

3- Growth and maintenance of *Fusarium* isolates

The isolates of *Fusarium* are grown in Petri dishes that contain PDA and incubated for 5 days at 25 0C. PDA is poured in glass tubes (volume 50 ml) as 20 ml for each tube and left until solidified. The medium is inoculated with *Fusarium* which is taken from the edge of recent produced colonies. Tubes are incubated at 25 ^{0}C for 5 days and then kept in refrigerator at 5 ^{0}C.

A- Study the effect of some ecological factors in the growth of five species of *Fusarium*

1- Temperature: Various temperatures are arranged (10, 15, 20, 25, 30, 35, 40 and 45 ^{0}C). Sterile Petri dish (diameter 8.5cm) contains 20 ml PDA for each is used; The *F. oxysporum* species are inoculated at the center of the agar plates by taken fungal disc (0.5 cm) from the edge of recent formed colonies. Plates are divided to five groups and incubated for 6 days (4 replicates for each isolate). Radial growth (colony diameter) is estimated on agar plate at 2 days interval (Gupta *et al*., 2010).

2- pH: Various pH are arranged (4.5, 5.5, 6.5, 7.5 and 8.5) for growth of *Fusarium* species. Similar steps in temperature are used. Radial growth was estimated on agar plate at 2 days interval

3- Light and Darkness: Effect of light and darkness on growth of *Fusarium* species is investigated by using (continuous light and continuous darkness). All other steps are applied as mentioned in temperature steps. Radial growth is estimated on agar plate at 2 days interval.

4- Culture media: Different culture media are used (PDA, SDA, and CDA) for growth of *Fusarium* species. Also similar steps are used as mentioned in temperature. Radial growth is estimated on agar plate at 2 days interval.

B- Detection of extracellular enzyme activities in five species of *Fusarium*

1-Production of Protease Enzyme:-

Proteolytic activity is detected by casein hydrolysis on agar plates containing milk agar media, the plates are incubated at 28 ^{0}C for 3 days .The enzyme activity is indicated by the formation of clear zone around colonies of *Fusarium* species.

2-Production of cellulose enzyme

Cellulase enzyme is detected by on agar plates containing CMC as substrate for growth, the plates are incubated at 28 ^{0}C for 5 days. The enzyme activity is detected by observing the clear zone around colonies.

3-Results and Discussion

1- The effect of some ecological factors in the growth of five species of *Fusarium*:

Growth of *Fusarium* (*Fusarium oxysporum, Fusarium proliferatum, Fusarium sacchari, Fusarium solani, Fusarium globosum)* is varied according to the level of temperature, pH, type of media, and light system. It is ranged between 15-30 ^{0}C, while the increasing or decreasing temperature below 10^{0}C or higher than 30 ^{0}C causes retardation of *F. oxysporum* growth. Maximum growth of *F. oxysporum* and *F.solani* is observed at 25 ^{0}C 8 and 7.5 respectively after 6 day of incubation followed by 20 ^{0}C (6.9 cm). No growth is detected for this fungus at 10 ^{0} C as well as at temperature above 30 ^{0}C. The radial growth of *F. sacchari* and *F. globosum* is increased at 20 ^{0}C in the 6 day of incubation while the highest rate of *F. proliferatum* growth (8cm) is achieved at 30 ^{0}C in the 6 day of incubation (Figure 1& plate 2). These results are compatible with (Swanson *et al*., 1985) which indicate that the mycelium of *F.* oxysporum grows well at temperature ranged between 24- 28 ^{0}C. Miller, 2001 and Jacobes *et al*., 1998 report that the optimum temperature of various fungi species is 25 ^{0}C. Desai *et al*., 2003 reported that *F. oxysporum* reveals maximum growth at temperature 25 ^{0}C on PDA while temperature from 10 and 40 ^{0}C causes inhibition to mycelial growth.

The maximum growth of *F. solani* is occurred at 25 ^{0}C followed by 20 ^{0}C, this result agrees with (Hung *et al*., 2001) which mention that the highest growth of *F. solani* is observed at 25 ^{0}C, but the growth reduced sharply at 10 and 35^{0}C, no growth was recorded at 5 and 40 ^{0}C. Wakle *et al*., 2007 have also reported similar findings in the case of *F. coreuleum, F. sacchari and F. globosum* which grows well at 20 ^{0}C. The growth of *F. proliferatum* is observed at 30 ^{0}C. Comparable conclusion was obtained by (Marin et *al*., 1995), they mention that the optimum growth of *F. proliferatum* is occurred at 30 ^{0}C. High temperature over 45 ^{0}C causes denaturation of enzymes like cellulase (Fayyz *et al*., 2009), While the decline of temperature to 5 ^{0}C fairly reduces the exchange of solutes across the plasma membrane (Tanner, 1997).

The results (Figure 3 & plate 4) showe that pH has significant effect ($p<0.05$) in growth of *Fusarium* on PDA at temperature 25 ± 2 ^{0}C and pH (4.5, 5.5, 6.5, 7.5, and 8.5). Highest growth for all isolates of *Fusarium* species is obtained at pH 7.5 (8.5cm) and minimum at pH 4.5 in the eighth day of incubation. Growth of fungi is declined by increasing or decreasing pH levels from neutral point. This results agree with the conclusion of (Glen *et al* .2003) that the optimum growth of *F. solani and F. oxysporum* at pH 7.5. The pH 7 is supported the growth of *F. oxysporum, F. sacchari, F. globosum, F. proliferatum and F. solani*. On other hand, Digark and Eluk, 2001) reports that highest growth is produced at pH 5. The pH of culture medium is one of the determining factors for metabolism and biosynthesis of secondary metabolites. pH is related to permeability characteristic of the cell wall and membrane (Hansen.1968). Although some studies are revealed the growth of fungus in acidic conditions but in this study it is found that the *Fusarium* species grown well at ranged pH between 5-7.

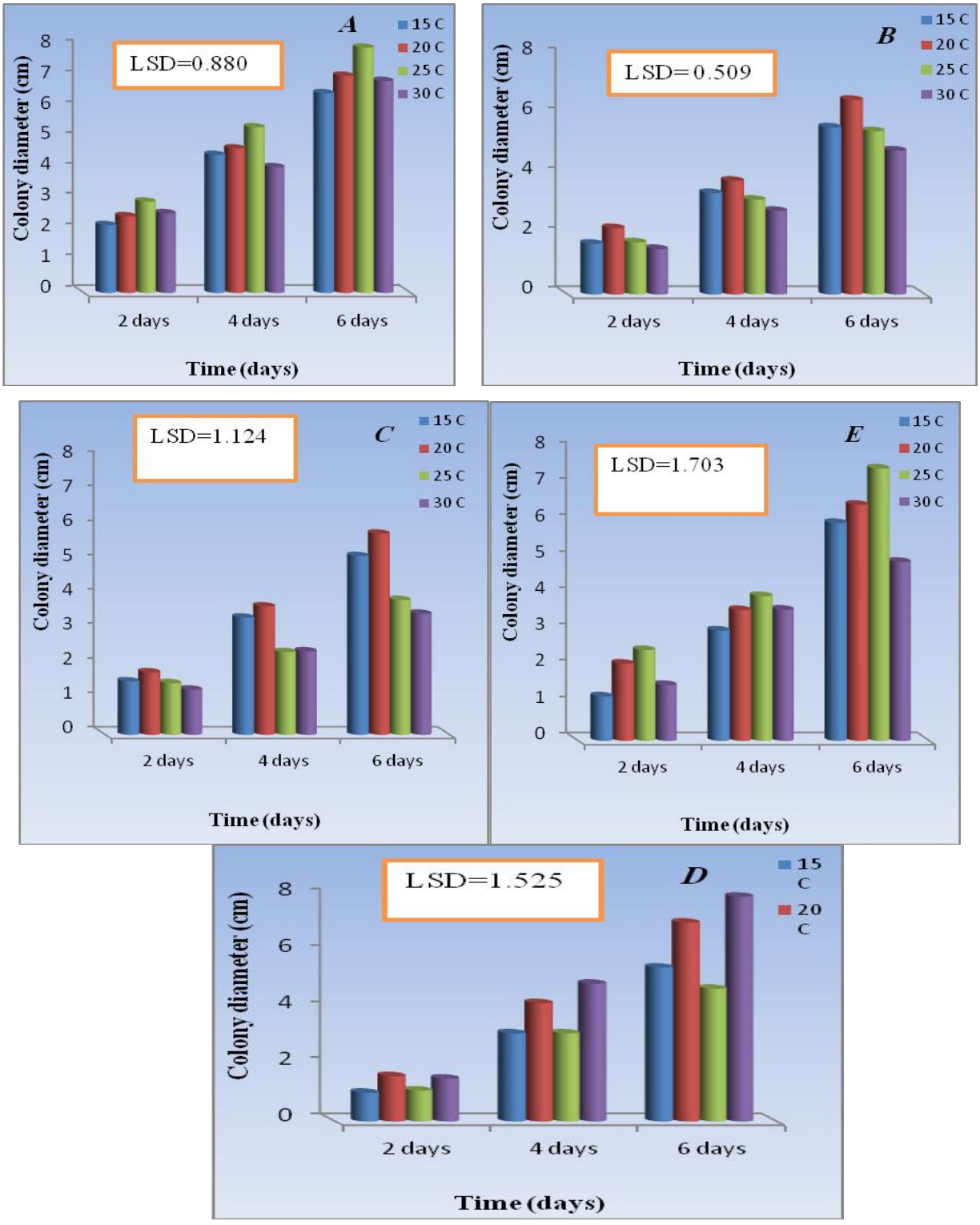

Figure 1: The effect of different temperature on the growth of *Fusarium* species (*A- F. oxysporum, B- F. sacchari, C- F. globosum, D- F. proliferatum and E- F. solani*) after six days of inocubation..(LSD = 0.05).

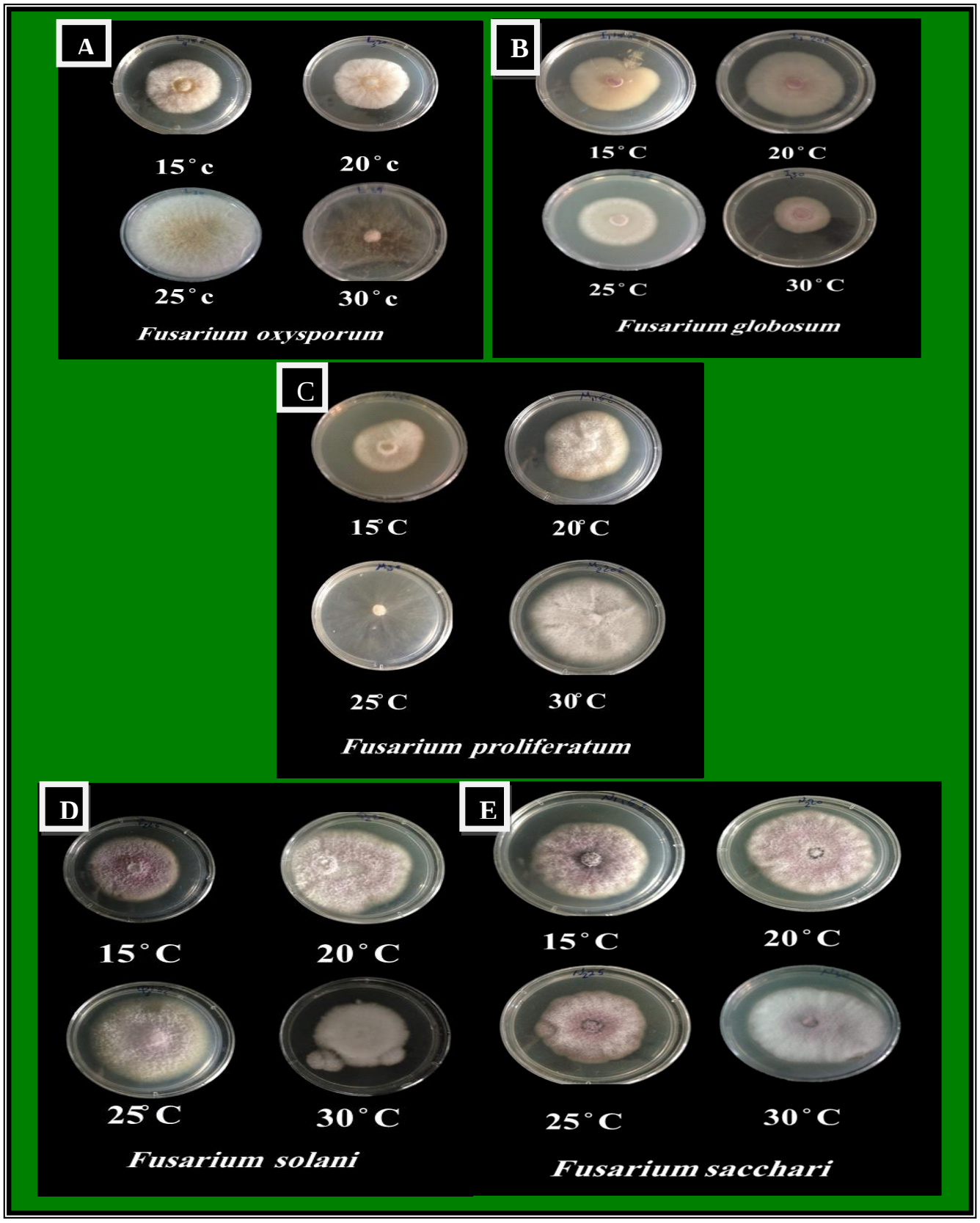

Plate 2: The effect of different temperature on the growth of *Fusarium* species (A- *F. oxysporum*, B- *F. globosum* , C- *F. proliferatum*, D- *F. solani* and E- *F. sacchari*)

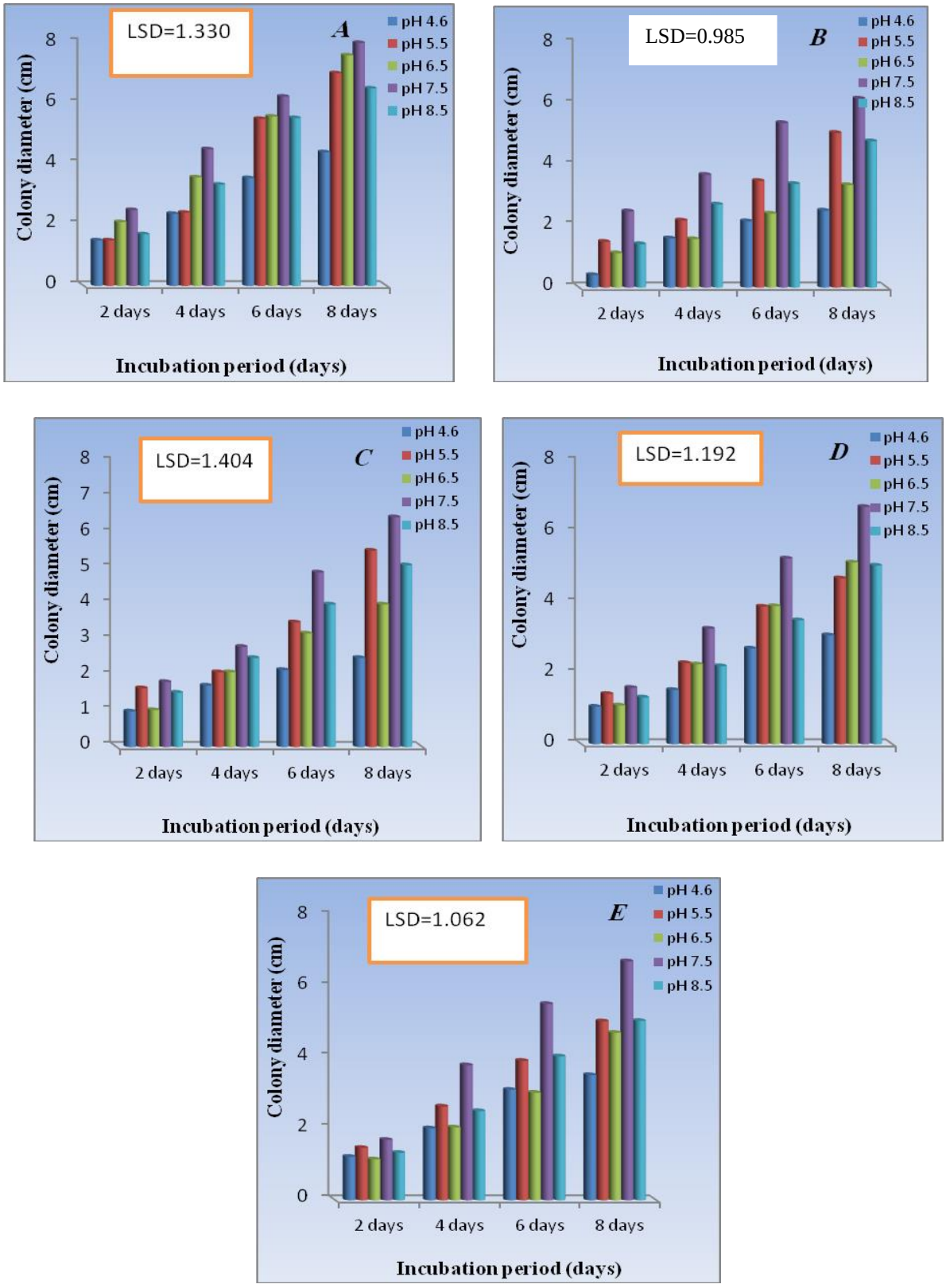

Figure 3: The effect of different pH on the growth of *Fusarium* species (A- *F. oxysporum*, B- *F. sacchari*, C- *F. globosum*, D- *F. proliferatum* and E- *F. solani*).(L.S.D= 0.05)

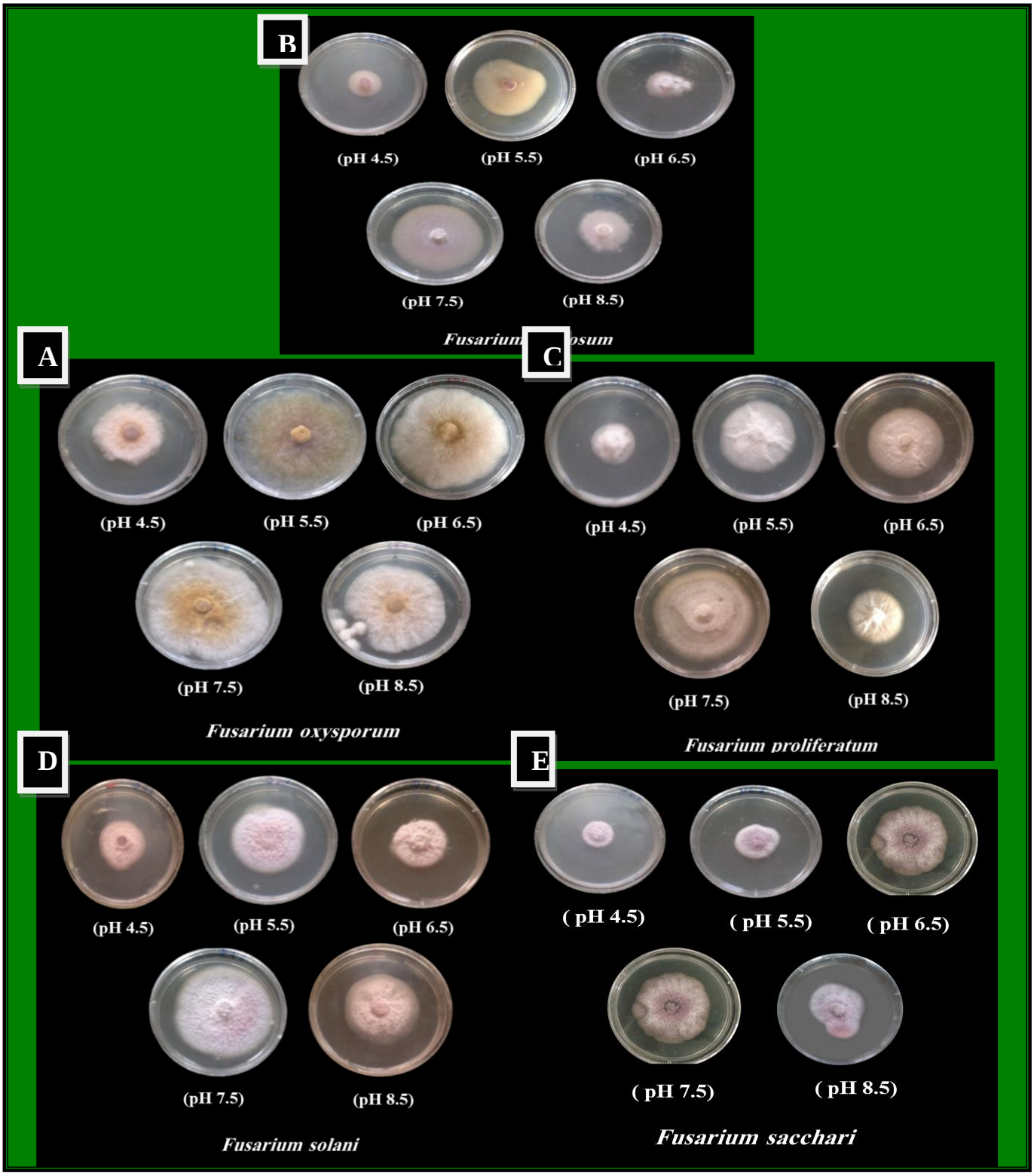

Plate 4: The effect of different pH on the growth of *Fusarium* species (A- *F. globosum*, B- *F. oxysporum*, C- *F. proliferatum*, D- *F. solani* and E- *F. sacchari*).

Growth of *Fusarium* species is determined under different light systems at temperature 25 ± 2 and pH 7.5. The results (Figure 5 & plate 6), revealed that the highest growth for all isolates of *Fusarium* species is occurred at darkness after 4 day of incubation. These results are compatible with (Fayzalla *et al*., 2008) results which showed the optimum growth of *Fusarium oxysporum* and *Fusarium solani* at continuous darkness or continuous light. Light has little influence on mycelial growth. However, there is little variation in mycelial growth under different light regime.

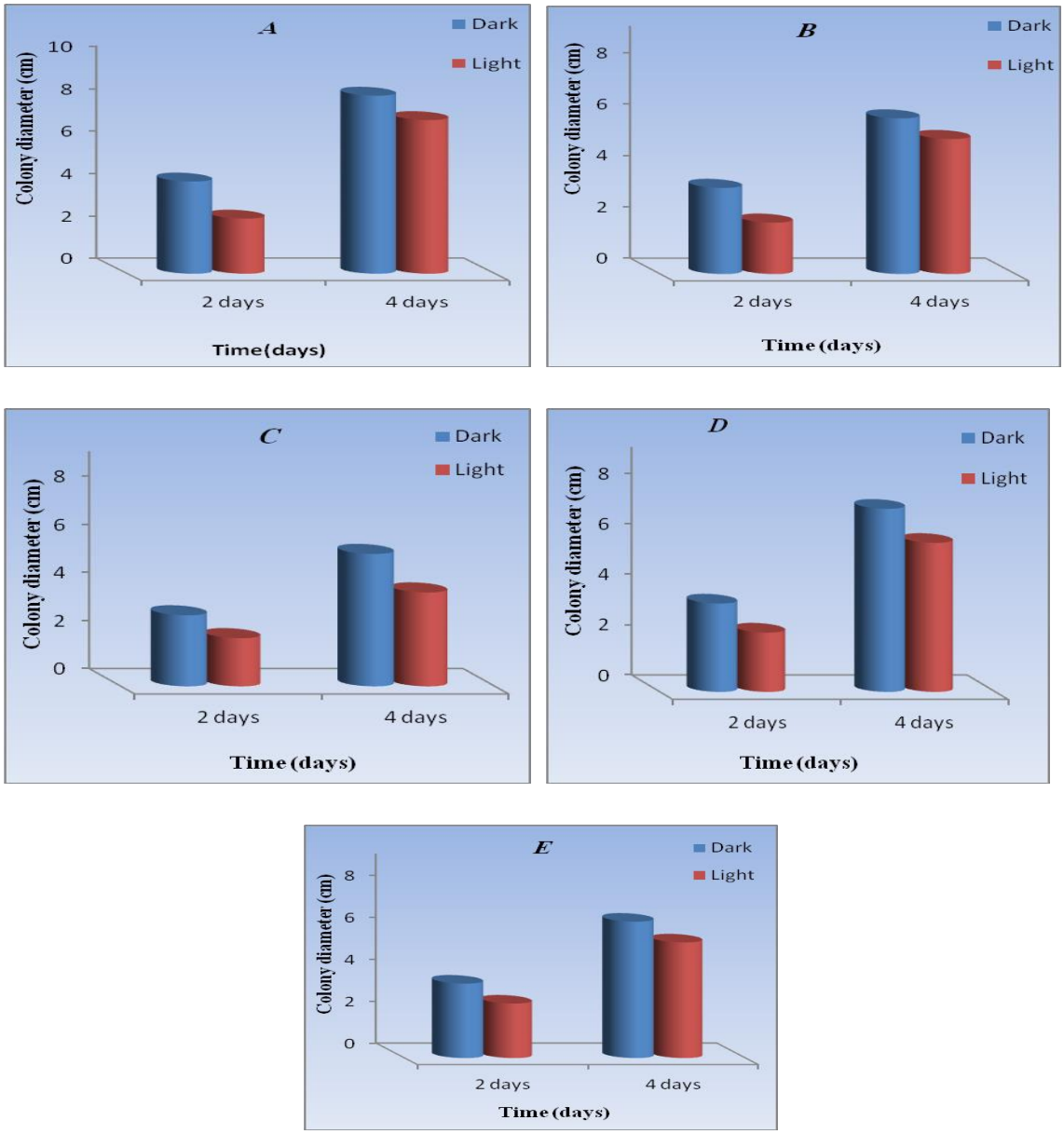

Figure 5:- The effect of light and darkness on the growth of *Fusarium* species (A- *F. oxysporum*, B- *F. sacchari*, C- *F. globosum*, D- *F. proliferatum* and E- *F. solani*) .

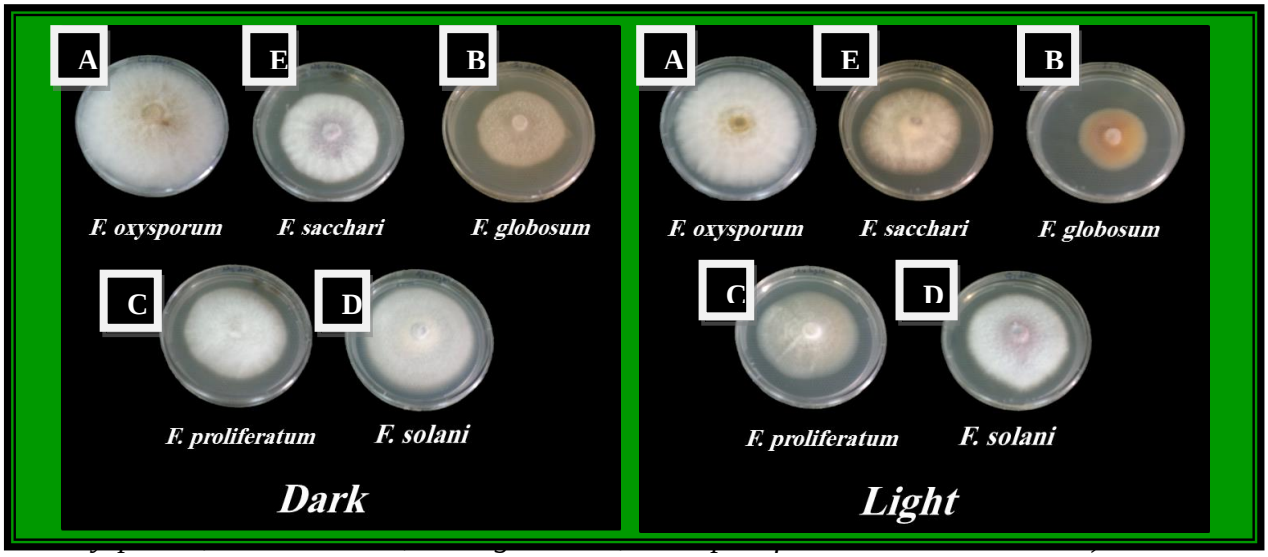

Plate 6: The effect of light and darkness on the growth of *Fusarium* species (A- *F. oxysporum*, B- *F. sacchari*, C- *F. globosum*, D- *F. proliferatum* and E- *F. solani*).

Type of culture media (SDA, PDA, and CDA) significantly ($P<0.05$) affecte the growth rate of *Fusarium* (Figure 7 & plate 8). Maximum growth of *F. oxysporum* and *F. solani* are recorded on SDA followed by CDA at the 6 day of incubation. *F. sacchari*, *F. globosum* and *F. proliferatum* showe maximum growth on CDA at temperature 25± 2 and pH 7.5. SDA medium is found to be the most effective culture medium for supporting the maximum growth of *F. oxysporum and F. solani*, because this medium contain, nitrogen, potassium and phosphorus which provide the fungi with necessary growth requirement. In contrast minimum fungal growth for these species is observed on PDA. (Farooq *et al*., 2005) finds that CDA and CSMA media are the best for mycelial growth of *Fusarium* wilt. These results differ from (Dikkar and Deshmukh, 2003) results who find that PDA is the best for *F. oxysporum* growth. While agree with their results the optimum growth of *F. sacchari, F. proliferatum, and F. globosum* gives the best growth on CDA due to nutritional requirements for growth of fungus in this medium such sodium nitrate as nitrogen source and sucrose for carbon source and potassium phosphate for phosphorus. .

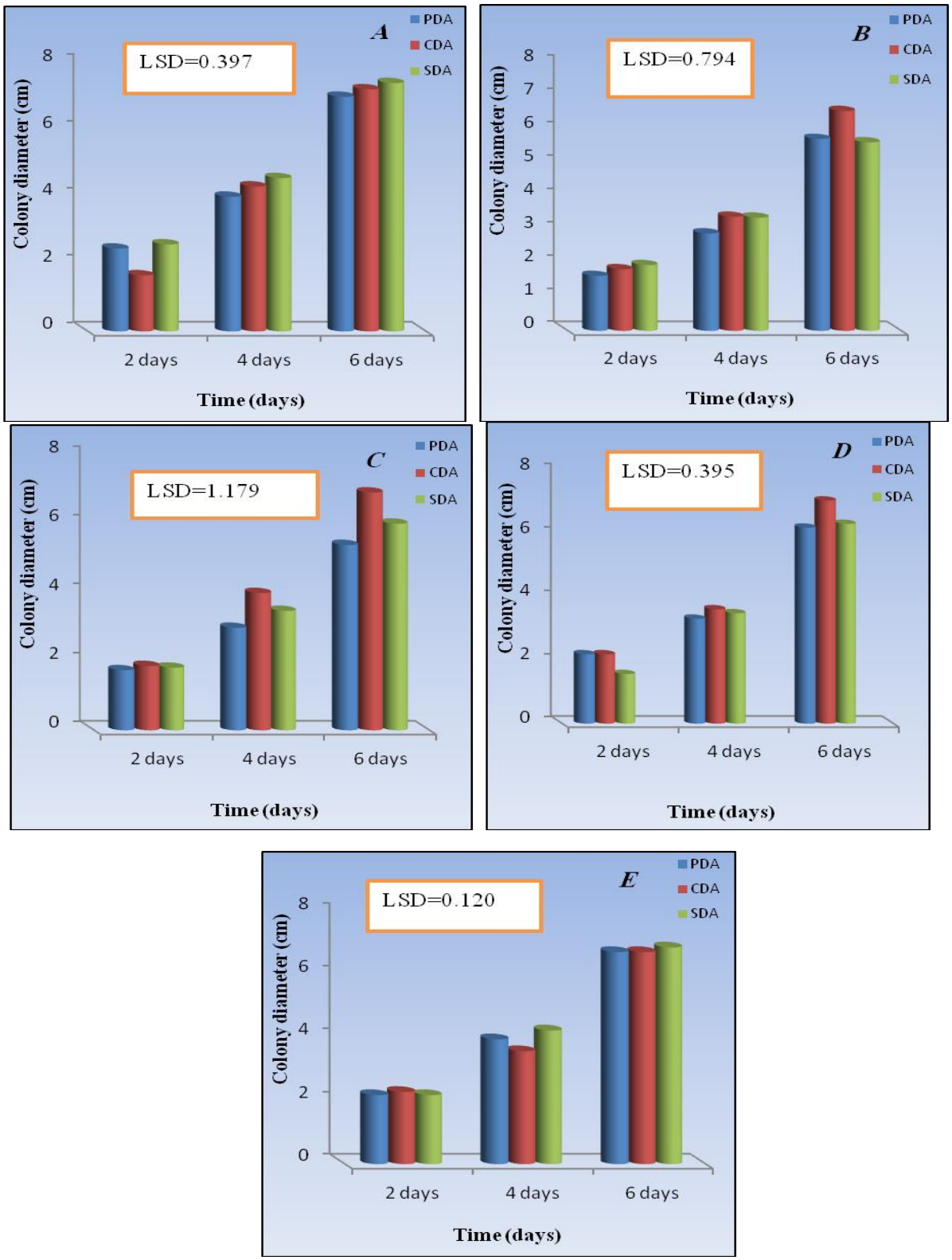

Figure 7: The effect of different media on the growth of *Fusarium* species (A- *F. oxysporum* , B- *F. sacchari*, C- *F. globosum*, D- *F. proliferatum* and E- *F. solani*) (L.S.D= 0.05).

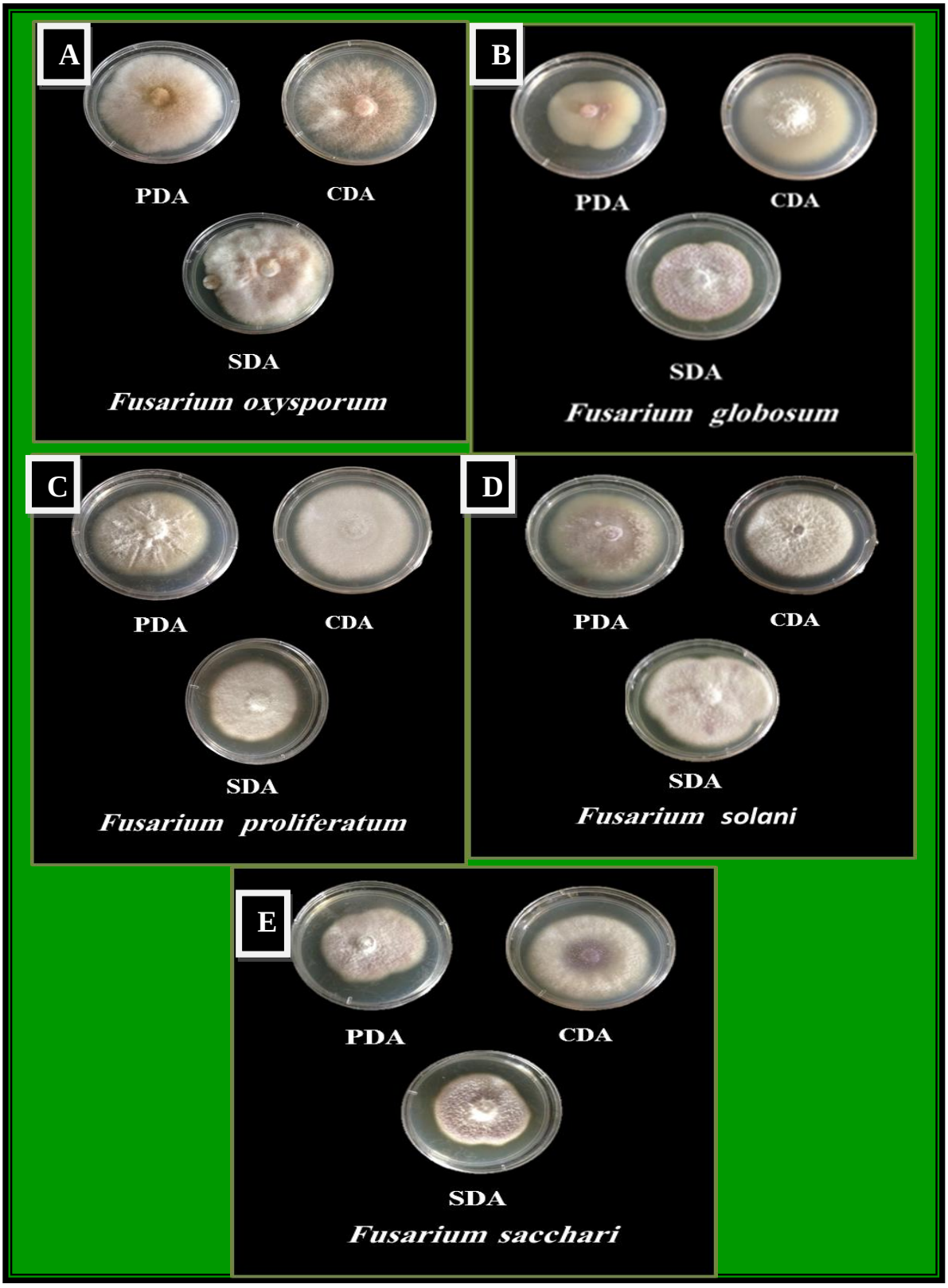

Plate 8: The effect of different media on the growth of Fusarium species (A- *F. oxysporum*, B- *F. globosum*, C- *F. proliferatum*, D- *F. solani* and E- *F.sacchari*).

2- **The Detection of External Enzymes:**

The results of this experiment (Figure 9), reveals clear zone for protease around colonies of *F. oxysporum* (6.1cm), followed by *F. sacchari* (5.8cm). Other isolates *F. solani, F. proliferatum and F. globosum* also give disintegration but less than that in *F.oxysporum and F. sacchari* at third day of incubation.Casein hydrolysis showed a clear zone around , characterizing them as proteolysis .Efficient protease activity has been described for *Fusarium* species. Some genus of microorganisms have already been studied for proteolytic activity such as *Pencillium, Fusarium, Bacillus and pseudomonas* (Barata *et al*., 2002; Kitano *et al*., 2002; Uyar and Baysal, 2004) . Nganga *et al* 2011 reports positive protease activity from *Fusarium oxysporum*, *Fusarium solani*. This study shows similar results with Amirita *et al*., 2012 about the protease activity by *Colletotrichum. carssipes, Colletotrichum .falcatum, Colletotrichum gleosporiodes.*

Table 1: Screening and hydrolysis of external enzymes activity by fungal isolates

Name of isolate	Protease enzyme	Celuulase enzyme	Chitinase enzyme
F.oxysporum	+	-	+
F.sacchari	+	-	+
F.globosum	+	-	+
F.solani	+	-	+
F.proliferatum	+	-	+

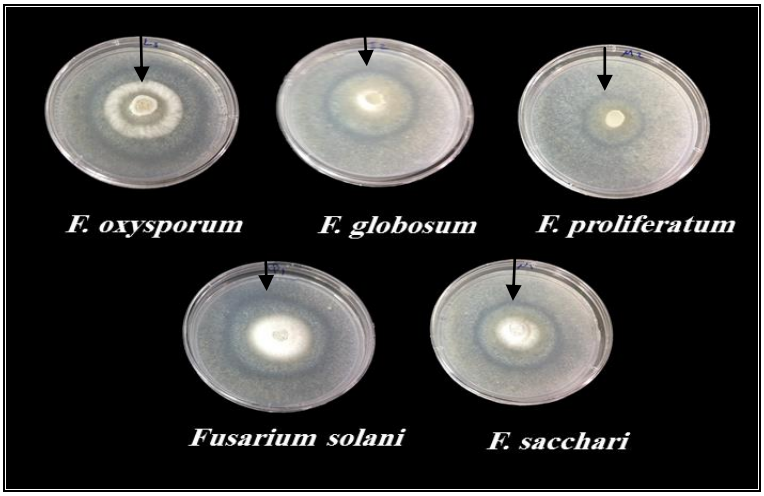

Figure 9: production of protease enzyme by *Fusarium* specieson skim milk agar after 3 days of incubation (*F. oxysporum,- F. globosum, , F. proliferatum F. solani* and *F. sacchari*).

. Cellulase enzyme was not detected in five species of *Fusarium* that used in this study. There is no activity for all species of *Fusarium.* Yoon *et al.,* 2007 successfully detects cellulose in *F. solani* using CMC agar but notices there is not cellulase activity not only from *F. solani* but also from other *Fusarium* species. These results are similarity to our studies. Cellulase is not common extracellular enzyme in *Fusarium*.

Conclusion

The present study concludes that temperature from 20 to 30˚C, pH 6 to 7, continuous dark, and culture media SDA and CDA are suitable for the radial growth of five species of *Fusarium*. The radial growth of resistant isolate was higher than that of the sensitive one. Environmental factors substantially affect the growth metabolism of the pathogen. Amounts of characteristic enzymes produced by fungi would be useful for selecting organisms best suited for industrial requirement.

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