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ROLE OF CERCOSPORIN TOXIN IN INDUCING SUGAR BEET LEAF SPOT

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ABSTRACT

Cercospora leaf spot is one of the most important widespread and destructive fungal diseases affecting sugar beet. The ability of most *Cercospora* isolates to cause disease is correlated with cercosporin toxin production. The yellow toxin first called (GF) for Gelben Fraktion, but now called (CBT) for *Cercospora beticola* toxin .In vitro preliminary experiments showed the ability of some *Cercospora* isolates to form red and yellow pigments in culture media. Applying pigments exogenously to leaves, showed its toxicity to sugar beet (*Beta vulgaris* L.), whereas, cercosporin necrosis lesions were similar to that lesions induced by the natural fungal infection. Factors influencing cercosporin in culture have been studied. Results indicated that PDA medium was the most favorable medium for overall growth. Comparison of measured amounts of cercosporin produced on different media confirmed that malt medium was the most favorable for (CBT) production in all tested isolates, on which the highest level of cercosporin was harvested. Light also plays an important role in growth, sporulation and toxin accumulation. The present study was concerned with the role of cercosporin toxin in inducing sugar beet leaf spot as well as the impact of bacterial toxin degradation as antimicrobial agent in disease control. The most efficient degraders were isolates of *Xanthomonas campestris* pv. *pruni* or *zinniae*.

Keywords: *Beta vulgaris* L., *Cercospora beticola* Sacc., cercosporin, *Xanthomonas campestris*.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is one of the most important sugar crops, grown all over the world .It supplies more than 40 % of the world's sugar. Egypt depends mainly on sugar cane to fulfill its demand of sugar. Recently, in Egypt, sugar beet comes second to sugar cane, i.e., its cultivation was established, especially in newly reclaimed lands, e.g., of Kafr El-Sheikh Governorate and El-Beheira (West Nobaria region) to cover the increasing sugar demand (El-Kholi, 1984 and El –Zahabi, 1987). The cultivated area was increased during the past 10 years 1990 – 2000 in which it was 40,800 – 105,600 Fadden, however, the total production of sugar beet was also increased from 789,000 – 2560,000 MT (FAO,2000).Sugar beet is affected by numerous diseases. Cercospora leaf spot is one of the most important, widespread and destructive fungal diseases affecting sugar beet. Spots usually appear on the older leaves. Leaves appear speckled from the small, brownish spots with reddish-purple borders. Eventually as the spots enlarge , their center drops out and the leaf dies. Yield and sugar content are reduced as a result of fallen leaves. The ability of most *Cercospora* species to cause disease is correlated with cercosporin production. Cercosporin is a photosensitizing perylenequinone toxin that destroys cell membranes of infected tissues. (Steinkamp, *et al.*, 1981and Yamazaki and Ogawa 1972)

.Toxicity of cercosporin or cercosporin derivative to many organisms including bacteria ,algae ,yeasts ,higher plants and mice as well as the nature of its photodynamic effects have been evident (Lynch and Geoghegan ,1975 Gavallini ,*et al.*, 1979 and Yamazaki ,*et al.*, 1975).The ability to manipulate cercosporin accumulation in specific isolates of *Cercospora* in culture is a necessary prerequisite for studying the regulation of toxin accumulation at a molecular level. Jenns,*et al.*,1989 studied media effects on cercosporin production and growth of isolates of *C. asparagi, C. beticola, C. kikuchii, C. nicotianae and. Zeae maydis*. Six growth media have been examined to select the favorable one for cercosporin accumulation. They also reported that both malt and potato-dextrose agar were generally favorable for toxin production. Steinkamp *et al.*, 1981 mentioned that *C.beticola* was cultured on PDA medium under fluorescent light at 15°c to obtain the mycelial mat and for cercosporin extraction trails .On the other side sporulating cultures of *C. zeae-maydis* were obtained by homogenizing freshly isolated or stored cultures in water, dispensing the homogenate on V-8 juice agar. Decoction media made from green or senescent corn leaves also was good for sporulation (Beckman and Payne 1982, Beckman and Payne 1983 and Wang *et al.* 1998). Lesions induced in leaves of *Beta vulgaris* 5 days after application of cercosporin produced by the fungus *Cercospora beticola* were compared with a published report of lesion incited by the fungus. Generally, it was found that lesions appears by the toxin effect, resembled in both size and appearance the natural infection, however, they were often more irregular in shape (Steinkamp *et al.*, 1981). Cercosporin accumulation also was regulated by temperature in four of the eight *Cercospora* isolates at 20°c than at 30°c. Malt media showed the highest level of cercosporin production (Jenns *et al* 1989). Mitchell and Daub have reported degradation of cercosporin toxin as a strategy for controlling cercospora disease, 1998 and 1999, whereas several *X.campestris* isolates representing two *pathovars* show efficient degradation activity. These cercosporin-degrading isolates are resestant to higher levels of the toxin than non –degrading isolates. The bacterial genes coding for the cercosporin degrading phenotype are being identified using Tn5 transposon and UV mutagensis technologies. The present study goal is studding the role of cercosporin toxin in inducing sugar beet leaf spot and how to detoxify that toxin which has an important agent in pathogenicity.

MATERIALS AND METHODS

Isolation of Fungal Pathogen:

Leaves exhibiting necrotic gray leaf spot lesions were collected from sugar beet –production region throughout Kafr El-Sheikh Governorate and Nobaria region Beheira Governorate,Egypt during 2000-2001 growing season. Single, well-separated lesions were excised, surface- sterilized and incubated on moist filter paper at 24°C for 24-to48 h to allow sporulation. *Cercospora* conidia were removed and transferred to vials of sterile distilled water .The conidial suspension was spread over the surface of potato dextrose agar (PDA) and incubated at 25 °C for 2 days, thin single colonies were transferred to fresh medium.

Effect of Medium on Growth and Cercosporin Production:

Six growth media as malt media (MALT), Cercospora sporulation medium (CSM), V-8 juice agar, Complete medium (CM), modified fries medium (MFM) and potato dextrose agar (PDA) were examined to determine conditions favorable for *C.beticola* growth (Booth, 1971).

Media: (MALT) medium contained 15g malt extract, 3g peptone, 30g glucose and 15g agar per liter. (CSM) contained 1.6g leucine, 5g sugar, 3.6g yeast extract, 0.5mg thiamine, 10mg biotin and 18g agar per liter .V-8 juice agar contained 19g Difco agar, 4g$CaCO_3$ and 300ml V-8 vegetable juice (Campbell Soup Co., Camden, NJ USA) per liter. (CM) contained 1g Ca $(NO_3)_2$ $4H_2O$,10ml of a solution containing (2g KH_2PO_2, 2.5g $MgSO_4.7H_2O$ and 1.5g NaCl in 100ml H_2O, adjusted to pH 5.3 with NaOH), 15g agar per liter and 10g of glucose. Modified Fries medium contained 30g sucrose, 5g NH_4 tarterate, 1g NH_4NO_3, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 1.0g NaCl, 0.13g CaCl $.2H_2O$, 1.0g Difco yeast extract and 15g agar per liter. (PDA) contained 24g Bacto dehydrated potato dextrose broth and 15g agar per liter. Plugs (6 mm diameter) taken from the margin of the tested isolate of *Cercospora* growing on PDA medium were placed in the center of Petri plates containing 25 ml of each of the six tested media, then incubated at 25 °C in an incubator with 16 hr of light and 8 hr of dark. Five plates were used for each medium .The diameters of the cultures area were measured every 3 days, through out 30 days of the experiment.

Effect of light on spore production:

PDA medium was distributed into nine petri dishes. Three plates were seeded with three-separated inoculums preparations. Inoculated plates were enclosed in plastic bags in stacks of three with two unseeded plates on top for shading. Incubation temperature was 25°C under diurnal fluorescent light (12 hr of light and 12hr of dark) for 14 days or under constant light for 11 days followed by 3 days of darkness or in complete darkness for 14 days .The combined spores from the two bottom plates of each stack were harvested with 10 ml of water and counted with a hemocytometer (Beckman and Payne, 1983).

Cercosporin assay:

Four plugs (6-mm diameter) were taken from 10 days old cultures of each of the six growth media of the tested isolate of *cercospora*. These plugs were either soaked in 8 ml of 5NKOH for 4 hr in the dark, after that, they were removed with the wide end of sterile Pasteur pipits. The absorbance of the soaked solution was measured at 480 nm in a Backman model 25 spectrophotometer, or they were ground with sand in 8mL of acetone, filtered and the absorbance read at 473 nm (the visible absorbance maximum of cercosporin in acetone). An exhaustive determination procedure has been done, where 100uL each of the extracts in 5N KOH and in acetone were subjected to thin-layer chromatography on silica gel 60 plates (Merck), pretreated in 2%H3PO_4, and dried overnight at 60°C,using hexane:

isopropanol (8:2) as a solvent. Spots with the same Rf as the cercosporin standard (SIGMA Chemical Co.) were scraped off the plate, resuspended in 2ml of acetone, and their absorbance read at 473 nm (Jenns *et al.*, 1989).

Application of cercosporin:

B.vulgaris: Glorria, Oscar, Pleno and Top cultivars with an intermediate level of field resistance to *C.beticola* leaf spot disease were grown by seeds in sterilized soil in a greenhouse. Extracted cercosporin solution was applied with a microsyring in 1 μ l droplets to adaxial surface, away from major veins, on mature leaf blades. Similar droplets of ethanol were applied to the other half of the same blades. Leaf tissues at the centers of the ethanol control droplets as well as some of the cercosporin droplets were pricked with the syringe needle to facilitate entry of materials into the leaf. The same treatment has been repeated using the standard compound of cercosporin whereas droplets of ethanol were applied to the other half of the same blades as check treatment. Immediately after treatment, plants were placed in a mist chamber for 5 days at 30°C, 90-100% relative humidity, and 16 hr of fluorescent light daily. Five days after treatment, leaf tissues containing chlorotic or necrotic lesions of depressed areas at the sites of both extracted and purified cercosporin application were removed, observed and compared with that ethanol control area according to (Steinkamp *et al.*, 1981).

Inoculation:

C.beticola conidial suspension was either atomized or brushed with a camel's hairbrush on both leaves surfaces containing 50 conidia per milliliter then kept under moist condition in the green house.

Antagonistic study:

Pure cultures of both *X.campestris* pv.*pruni* and *X.campestris* pv.*zinniae* were maintained on King *et al*,'s B agar (KB) medium for 24hr at 25°C. The inoculation suspension of the bacteria was applied at 25°C in darkness for 36hr .The concentration of the bacterium suspension was 10 cfμ/ml. Fifty μl of bacterial suspension ($2x10^2$ cfμ/ml) were streaked on PDA, KB and on plates with two layers of KB and PDA media (sandwich layers of 2mm thick) plates. Discs (2mm diameter) of mycelial mat of *C.beticola* were taken from the growing edges of fresh pure culture and placed in a central well on the plate. The plates were incubated under alternating cycles of 12 hr of near ultraviolet light and 12hr of darkness then observed after 6 days.

Cercosporin degradation:

Twenty –five ml of malt liquid medium in 150-ml Erlenmeyer flasks, were inoculated with 0.5ml spore suspension of *C .beticola* in three replicates each.The cultures were incubated at 25°C, then six days later, fifty μl of each bacterial suspension were added to the fungal inoculated culture medium. The cultures were incubated for another 6 days, and then cultural filtrates were separated. Peak levels of cercosporin were measured after 12 days. Check treatment received 50 μl of sterile water to the fungal culture medium

instead of bacterial suspension. Cercosporin extraction was carried out according to the method mentioned before by Jenns *et al.*, 1989.

RESULTS

Cultural characteristics:

C.beticola Sacc. Isolates were obtained from single typical necrotic leaf spots, which are nearly circular. Lesions tan to light brown with dark brown to reddish borders. Stomata seemed to be as minute black dots in the necrotic centers of the spots. Necrotic spots become gray. Conidiophores are unbranched, growing from stomata, pale brown near the base but hyaline near the apex, sparingly sepetate. Conidia are straight to curved, needle – shaped, hyaline and have three to14 septa in culture. Vegetative growth on PDA consisted of a dark –gray mycelium producing a reddish purple pigment after 5-7 days of growth in light.

Media effects on cercosporin production and growth:

When different growth media have been tested the results showed that: several isolates of *cercospora* produced less red pigment assumed to be cercocporin toxin (CBT), when grown on CSM than when grown on any other growth medium tested. Omission of individual ingredients of the medium, however, revealed that no one component was responsible for pigment suppression. Addition of yeast extract, sucrose or thiamin were partially restored the red pigmentation in which accumulated cercosporin levels varied among these media and isolates. Comparison of measured levels of (CBT) produced on different growth media CM, CSM, MFM, PDA, V-8 and malt confirmed that CSM was unfavorable for toxin production .On the other side the most favorable medium for growth of the tested isolates was PDA (fig. 1). However, malt medium gave the highest level of (CBT). Averaged over all isolates showed that both malt and PDA were more favorable for (CBT) accumulation and complete medium came next to them compared with the other three tested media. However, peak level of cercosporin measured after 6-8 days on malt. CBT levels increased gradually by time, but a decreasing has been observed after 20 days of the experiment. Whereas, levels of cercosporin on malt remained fairly constant (Fig 2).

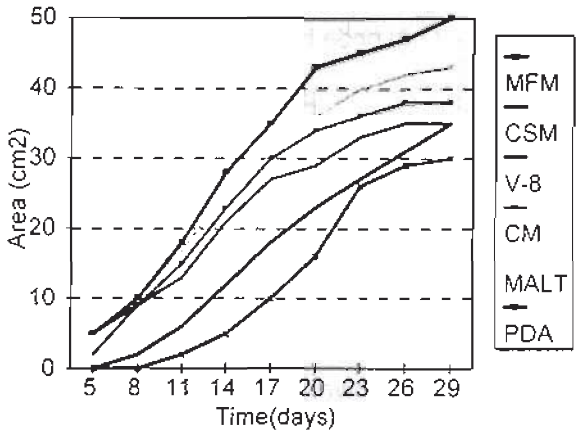

Fig.1- Mean increase in colony area over time for *C. beticola* on six media.

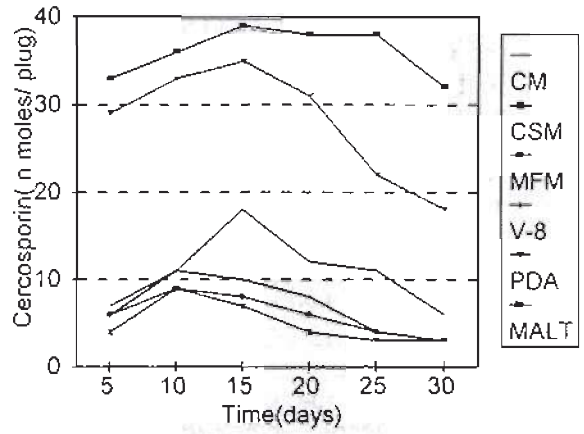

Fig: 2- Mean of cercosporin production levels (n mol) over time (days) by *C.beticola* on six media.

Light effects on sporulation:

Three light regimes were compared for their effect on spore production of *C. beticola*. An interaction was also found between medium and light regims (Fig.3). Under diurnal light, sporulation was relatively low on PDA but abundant on CSM and V-8 medium. Under constant light followed by 3 days of darkness, CSM medium was the best one for sporulation; however, all other media showed some decreasing in sporulation. Cultures incubated under complete darkness were found to be less in number of spores. Although spore production was greater on CSM medium than on V-8 juice under constant light followed by dark, but colonies seemed to be more aerial and fluffy. Results indicate that both medium and light influenced spore

production by C. *beticola*, but a greater number of spores has been supported more under diurnal light on different growing media than did under constant light followed by dark or under complete constant darkness.

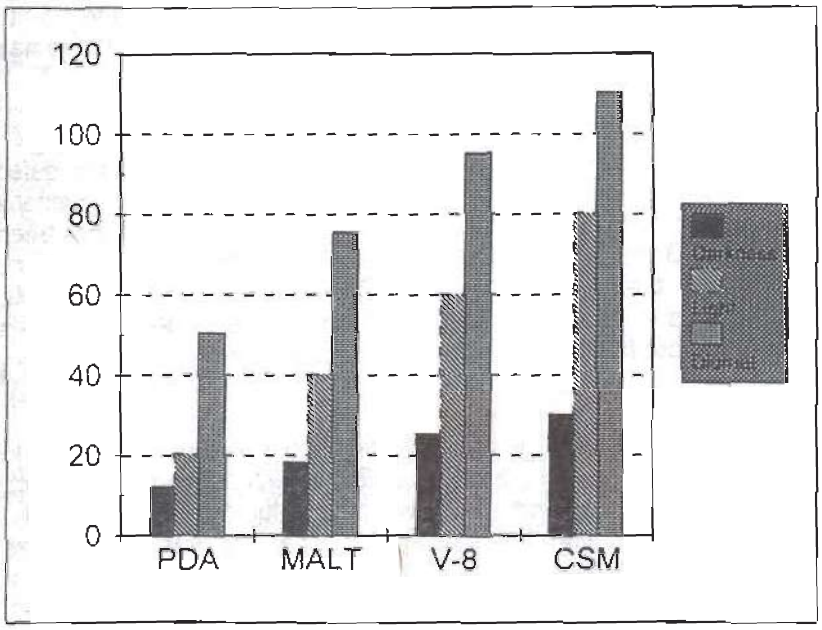

Fig (3): Effect of three light regims on sporulation of *C. beticola* harvested from different growing medium.

Cercosporin effects on plants:

Lesions with necrotic centers surrounded by somewhat chlorotic halls that graded into apparently healthy green tissue at the periphery were started to develop 5 days after cercosporin was applied. Individual leaf spots were nearly circular, measuring 3-5 mm in diameter at maturity. The spots were light brown with dark brown to reddish borders similar to that induced by the natural fungal infection, although the cercosporin-induced lesions were often more irregular in shape. No macroscopic or microscopic lesions were found where ethanol droplets had been applied unless the site had been physically wounded at the time of application, in which cause a small necrotic area around the wound. Tissue immediately surrounding the necrotic area appeared healthy; as compared with untreated control leaf tissue. In pots, inoculated sugar beet leaves with isolated fungus of *C. beticola* showed the first macroscopic symptoms of the disease as chlorotic dots 10-13 days after inoculation under greenhouse conditions. Chlorotic dots enlarged and sometimes developed a watery or greasy appearance. Necrosis progressed gradually from the center of the chlorotic dots and all the lesions margins had turned to a tan or brown color. Conidia and conidiophores were produced 1-3 days after the lesion become necrotic and about 15-20 days after inoculation.

Symptom expression in mature plants in this study was typical of that expression in plants grown in the field. No consistent differences in susceptibility were observed among leaves on the same plant. Generally, initial macroscopic symptoms occurred 1-3 days earlier than in adult plants ,and conidiophores production observed 3-4 days earlier in younger plants Blighted leaves soon collapsed and twisted toward the ground but remain attached to the crown.

Antagonistic evaluation:

Data presented in this study showed that the growth of the tested fungus *C.beticola* was inhibited to a great extent by the antagonistic bacteria related to *X.campestris*. The expression of antagonistic relationship has been observed. On the KB assay medium, both *X.campestris pruni* or *X.campestris zinniae* expressed the highest level of antagonism, against *C.beticola*. However, antagonistic activity was less pronounced on PDA, which is resulted to be the most favorable medium for fungal growth.

Cercosporin degradation:

The present study revealed that certain bacterial agents correlated to *X.campestris* that have specific ability to detoxify cercosporin toxin product. Cercosporin level produced on malt liquid medium 6 days after the inoculation or before the addition of bacterial suspension was measured as 35 n moles of cercosporin and began to decrease until the twelfth day of incubation to measure as 7 n moles in the presence of the bacterial inoculum suspension. Thus the degradation activity is acted, a green breakdown compound accumulated in the tested growing medium.

DISCUSSION

Several factors influenced growth of *C.beticola* the causal agent of sugar beet leaf spot. The success of this fungus as a plant pathogen is highly correlated with cercosporin toxin production that destroys cell membranes of infected tissues. Our results are in agreement with those of Jenns et al., 1989 whom found that malt was the best tested media for cercosporin production and PDA was the most favorable for growth of *C. beticola*. Light also is known to be a strong regulator factor for sporulation and toxin accumulation, if all other conditions were held constant. There for light –grown cultures had fast growth and much higher levels of cercosporin than those grown in darkness. The present study explained also the observation of great number of mycelial fragments, which have been collected with harvested spores from CSM medium than on V-8 juice under constant light followed by darkness. Where the growing mycelium was fluffy, this is because CSM medium contained sugar, yeast and thiamine compounds, which partially restored the red pigment, as it assumed to be cercosporin toxin. However, CSM considered being unfavorable medium for CBT production. Such results support the study of Jennes *et al.*, 1989. Optimum sporulation had occurred under diurnal light, which agrees with the observation of Beckman and

Payne, 1983. Batchvarova *et al.*, 1992 and Ehrenshaf *et al.*, 1995 purified a red toxin of cercosporin secreted by the fungal mycelium of *C.oryzae* where the toxicity of cercosporin depended on illumination and they suggested that carotenoids are implicated in the resistance mechanism of *C.nicotianae*. The present work was initiated to determine the effect of cercosporin in forming necrotic lesions in sugar beet leaf tissue and to compare these effects with those in lesions produced by the fungus to clarify the role of cercosporin in host-pathogen interaction. The results indicated that obtained cercosporin from the tested fungus could induce lesions symptoms to plants, similar to that of the fungus-induced. This is in the line with Steinkamp *et al.*, 1981 who mentioned that some species of *Cercospora*, including *C.beticola* produce other secondary metabolites, which might act separately or interact with cercosporin in the production of symptoms in host plants. Rathaiah 1977 reported that extensive random growth of *C.beticola* Sacc. Germ tube on sugar beet foliage under continuous wetting. Beckman and Payne 1982 discussed the same condition where germ tube and its branches grew toward stomata before leaf penetration under high relative humidity.

Toxin degradation is considered a strategy for diseases control. Antagonistic study revealed that certain isolates of bacteria *X. campestris* proved to be promising in reducing the growth of *C.beticola* and consequently cercosporin toxin production in artificial sterile liquid medium. This finding supports the work of Mitchell and Daub 1998&1999 who found that two pathovars of *X.campestris* play an efficient role in toxin degradation activity. The result cleared that the most effective degraders detoxify >80% of cercosporin toxin in growth medium within 110 hours. This finding is in close agreement with that obtained by Michail *et al.*, 1994 as certain natural and chemical materials were tested to detoxify aflatoxins produced by *Aspergillus flavus* Link, isolated from paddy grains.

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دور السم الفطري سركسبورين في أحداث مرض تبقع الأوراق لمحصـــول بنجـــر السكر

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تناول البحث دراسة مرض تبقع الأوراق السركسبورى في بنجر السكر الذى يعتبر مـــن أهم الأمراض الفطرية المؤثرة في زراعات بنجر السكر، حيث أتضح أن قـــدرة معظـــم عـــزلات السركسبورا في أحداث المرض متعلقة بما تنتجة هذه العزلات من السم سركسبورين والذي يرمـــز له بالأحرف (CBT) نسبة ألي الفطر المفرز له سركسبورا بتيكولا ، تباينت قدرة بعض العزلات في إنتاجها لصبغات حمراء وأخرى صفراء في بيئات النمو ،وبمعاملة أوراق بنجر الســـكر بـــهذه الصبغات ، أظهرت سميتها للنباتات بإعطائها أعراض مرضية مشابهة لتلك التي تحدثها العـــدوى الفطرية .

أهتم البحث أيضا بدراسة العوامل المؤثرة على النمو الفطري وأيضا على إنتـــاج الســـم الفطري معمليا (سركسبورين) حيث تم اختبار ٦ أنواع مختلفة من بيئات النمو فكانت بيئـــة أجـــار البطاطس (PDA)هي أنسبها لنمو الفطر ،بينما كانت بيئة المولت هي أفضلها فـــي إنتـــاج ســـم السركسبورين وكان للضوء دور كبير في تجرثم وانتاج السم الفطري ، كما أجريـــت اختبـــارات حيوية بهدف مقاومة الفطر ومحاولة لتقليل قدرته على إنتاج السم الفطرى، فعند معاملـــة الفطـــر بعزلتين من البكتريا زانثوموناس كمبسترس , أظهرت مقاومتها الحيوية تجاه النمو الفطري ، كمـــا أكدت قدرتها العالية في هدم وتحليل السم الفطري بنسبة ٨٠% تقريبا مما يؤهلها للاســـتخدام فـــي المقاومة الحيوية لهذا الفطر .