J. Plant Prot. and Path., Mansoura Univ., Vol. 3 (7): 657 - 666, 2012

EVALUATION OF SOME ASSAYS TECHNIQUES FOR DETERMINATION OF SUSCEPTIBILITY OF GARLIC CULTIVARS TO THE PINK ROOT DISEASE

Shalaby, S. I. M. ; S. M. A. Morsy and A. A. Abd El Baky
Plant Pathology Research Institute, Agricultural Research Center, Giza.

ABSTRACT

Biological and physiological assays have been used to evaluate garlic cultivars for their resistance to the pink root disease. Biological methods using artificial media amended with *Pyrenochaeta terrestris* and/or its toxins or using soil infested with the pathogen *P. terrestris* were tested to evaluate the resistance of seedlings of various garlic cultivars against the pink root disease. Results showed that the assay using the fungus-free Pyrenochaeta toxins was more reliable than using the fungus itself, in which determination of the disease resistance of garlic seedlings was possible in 7 days without the problems of contaminations. Physiological assays indicated that histones and RNA contents in garlic plants play a role in their resistance to the pink root disease.

Keywords: Garlic, pink root disease, *Pyrenochaeta terrestris*, disease resistance

INTRODUCTION

Pink root is one of the most important diseases that attack cultivated *Allium* spp. (Anonymous. 1973). Pink root disease caused by *Pyrenochaeta terrestris* has been reported as a serious disease to garlic in Egypt (Shalaby *et al*., 2002). Pink root pathogen is soil-borne fungus, which remains viable in the soil for many years (Rengwalska and Simon, 1986). Roots infected by *P. terrestris* turn pink initially and then become brittle and die. Although *P. terrestris* can be present in roots, it does not invade the basal plate or stem of the bulb (Coleman and Ellerbrock, 1997).

Crop rotation, solarization and fumigation are the most common methods for controlling soil-borne diseases (Porter *et al*, 1989). Although crop rotation is often suggested to decrease inoculua of soil-borne pathogens, the wide host range of *P. terrestris* makes this control strategy impractical. *P. terrestris* has been isolated from and has been shown to cause disease on many species of plants from 45 genera, including other *Allium* spp.**,** broadleaf weeds, grasses, grains and vegetables (Coleman and Ellerbrock, 1997). Solarization is not effective measure because garlic must be in the field throughout the cold months of the year in Egypt. Fumigation is expensive, and there is growing concern about the environmental hazards of chemical fumigants. Additionally, the high cost of registration for use on a minor crop such us garlic may limit fumigant availability in the future.

Resistant garlic cultivars offer one of the best non chemical means for controlling soil-borne diseases. Little garlic breeding programs have been directed toward developing pink root resistant material. All garlic in

commercial production today is a sexually propagated, but recent development suggested the possibility of routine sexual reproduction and consequent in corporation of new genetic variation into cultivated garlic (Konvicka, 1984). The present studies were undertaken to develop assays to evaluate pink root reaction with garlic cultivars.

MATERIALS AND METHODS

The pathogen:

Pathogenic isolate of *P. terrestris* was obtained by isolation from infested garlic in Ismailia governorate, Egypt. The cultures of various isolates of the fungus were identified according to Barnett (1960). Inoculum of the pathogen was prepared according to Rengwalska and Simon (1986). The soil (23.5 kg) in 25 cm diameter pots was infested with 100 ml of *P. terrestris* inoclum and 1.4 L of distilled water were then added to the soil to help in even distribution of the inoculum throughout the pot soil as well as to provide enough moisture to enhance the pathogen activity. Pathogenicity tests of *P. terrestris* were confirmed.

Toxin production:

Pyrenochaeta toxin(s) was(were) produced on Czapek's medium with powdered cellulose. Cultures of the respective isolates of the fungus were used to inoculate four liters of the broth medium (Hess and Weber, 1988). The culture filtrate containing the fungal toxin(s) was concentrated by evaporation at 45 C. One liter of concentrated culture filtrate was added to 1.2 L butanol and shaken. The solvent was allowed to separate into phases in a separatory funnel. The top (butanol) layer contained most of the toxic material. The bottom (water) layer was re-extracted with 800 ml butanol. The butanol fraction was retained and combined with the first butanol fraction. The combined butanol fraction (about two liters) was evaporated to dryness at 45^{o}C. The residue solubilized by addition of 20 ml ethyl ether and tested in a seedling assay.

Garlic Seedlings preparation:

Cloves of Balady, Seds-40, Chinese, American and Spanish garlic cultivars were germinated by soaking in distilled water on filter paper in Petri dishes (9-cm-diam) for 7-10 days. During that period, the water was changed several times. Garlic seedlings were used in the bioassay for determining their resistance to the pink root disease.

I. Biological assays:

Rating for pink root reactions was performed on garlic seedlings grown in artificial media amended with *P. terrestris* or with Pyrenochaeta toxins.

1. Using artificial medium amended with *P. terrestris*:

Previously germinated garlic seedlings of 5 cultivars (Balady, Seds-40, Chinese, American, and Spanish) were transferred to 9 cm Petri dishes containing artificial agar medium (1 g $MgSO_4$, 3 g $NaNO_3$, 20 g agar, and 1000 ml H_2O) (Hess, 1966), that was inoculated with *P. terrestris* and the surface of the agar was covered with the fungal growth. Sterile 9-cm-diameter

filter papers were placed over the seedlings and were moistened with sterile distilled water. The dishes were placed at room temperature for 10 days. Sterile distilled water was added periodically to prevent drying. Garlic seedlings placed in dishes containing fungus-free medium and treated similarly served as the control treatment. The treatments were distributed using a randomized complete block design with 20 replicates. Percentage of the pink root disease was estimated.

2. Using artificial medium amended with Pyrenochaeta toxins:

Pyrenochaeta toxins (as 0.2 ml of ether soluble fraction) were added to the agar surface in Petri dishes containing same agar medium as described above but with no fungus. After the ether evaporated, pre-germinated seedlings of various cultivars of garlic were placed on the agar surface and covered with filter paper moistened with 0.1 ml of the toxin as ether fraction. Sterile distilled water was used to moisten the agar surface and filter paper. The control treatment was similarly prepared but only sterile water was added, instead of toxin fraction, to the agar surface and filter paper. Seedlings were kept in the dishes at room temperature for 7 days. The Petri dishes of all treatments were arranged in a randomized complete block design with 20 replicates. Root lengths of seedlings were measured as an index of the toxicity of the fungal toxins.

3.– Using soil artificially infested with Pyrenochaeta inoculum:

Garlic cloves of five cultivars (Baledy, Seds-40, Chinese, American, and Spanish) were sown in pots (25-cm-diam.) inoculated with *P. terrestris* as previously mentioned in October at the rate of two cloves per pot. Pots of fungus-free soil served as control treatment. The treatments were arranged in a completely randomized design with 20 replicates per treatment. Percentage of the pink root disease was estimated 100 days after planting. Garlic bulb yield was determined as g/pot.

II. Physiological assays:

Garlic plants of Balady (resistant) and Seds-40 (susceptible) cultivars were used in this study. This experiment was carried out under greenhouse conditions. Pots (30-cm-diam) filled with *P. terrestris* infested soil as mentioned before. Cloves of Balady and Sed-40 garlic cvs were sown on October 3, 2010 at two cloves/pot. Pots of fungus-free soil served as a control. Pots of all treatments werearranged in randomized complete block system with ten replicates per treatment. Plant samples were randomly collected after 100 days from three pots. Higher molecular weight basic proteins [Pyrenochaeta histones (poly lysine and poly arginine)] were extracted from garlic shoots according to Johns (1964) and Bonner *et al* (1968), using electrophoretic analysis of basic protein fractions. RNA content was estimated in fresh leaves by the method described by Nitsan and Lang (1966). Free phenols were determined according to Anonymous (1960).

RESULTS AND DISCUSSION

I. Biological assays to evaluate garlic cultivars for their susceptibility to the pink root disease

1. Using artificial medium amended with Pyrenochaeta inoculum:

All garlic cultivars tested did not significantly differ in their susceptibility to the pink root fungus, when using artificial medium containing the fungus (Table 1). The fact that the disease incidence was relatively high on all cultivars may be due to that the inoculum load was so high that it was not a good measure to disease resistance of garlic seedlings. This suggestion can be elucidated in the light of the findings of Hess and Weber (1988) who indicated that non-pathogenic isolates of the fungus *P. terrestris* did not infect host tissue, although pink hyphae were sometimes observed on root surface.

Indication of the pathogenicity of the fungus was obtained within 10 days but the susceptibility was limited. Therefore, using artificial media amended with *P. terrestris* was not effective measure for pink root resistance determination of garlic cultivars.

Table (1): Screening of garlic cultivars for their susceptibility to the pink root disease by using artificial medium amended with Pyrenochaeta inoculum.

Garlic cultivar	Pink root disease incidence* (%), 10 days after transferring garlic seedlings onto the inculcated medium
Balady	62
Seds-40	65
Chinese	65
American	65
Spanish	65
L.S.D. (5%)	n.s.

*** Average of 20 seedlings for each treatment or cultivar. Control was considered to be 0% disease incidence.**

2. Using artificial medium amended with Pyrenochaeta toxins:

Data presented in (Table 2) show that there is a significant differences among cultivars in their susceptibility to the pyrenochaeta toxins. Cultivar seedlings of Balady showed the least degree of susceptibility. On the other hand, seedlings of Spanish cultivar showed intermediate reaction to the toxins, whereas seedlings of Seds-40, Chinese and American cultivars were highly susceptible to Pyrenochaeta toxins. This assay gave good indication of resistance/susceptibility of seedlings of garlic cultivars. *Pyrenochaeta terrestris* produces three pyrones known as Pyrenocine A, B, and C (Sato *et al*, 1981 and Sparace *et al*, 1984). Pyrenocine A inhibits seedling elongation and is toxic to tissue protoplasts, whereas pyrenocines B and C appear to have no effect. These metabolites may have a role in the pink root disease. It is clear that using Pyrenochaeta toxins was effective measure for pink root resistance determination of garlic cultivars.

Table (2): Screening of garlic cultivars for their susceptibility to the pink root disease by using artificial medium amended with Pyrenochaeta toxins

Garlic cultivar	Root length (mm)[1]		
	Untreated control[2]	Treated[3]	Inhibition (%)
Balady	3.2	3.0	6
Seds-40	3.6	1.7	53
Chinese	3.6	1.7	53
American	3.5	1.8	40
Spanish	2.9	2.1	28

[1] **Average of 20 seedlings for each treatment or cultivar.**
[2] **Control was treated with sterile distilled water only.**
[3] **Toxin concentration was 20 mg/ml.**

3. Using soil artificially infested with *P. terrestris* inoculum:

Symptoms of pink root on garlic plants were apparent 90 days after planting in infested soil. The pink root disease symptoms were measured on the basis of root pinking and basal plate rot as described by Nichols *et al*. (1965). There were great differences between tested cultivars in their resistance to the pink root disease (Table, 3). Balady was highly resistant as indicated by zero incidence of pink root. Spanish cultivar plants were less resistant than Balady's as 25% pink root incidence was recorded. Seds-40, Chinese and American cultivars plants were highly susceptible (69.18 , 69.18, and 58.30%, respectively).

The was a negative relationship between mean pink root incidence and mean yield of bulbs of the tested cultivars (Table 3). In inoculated plants, bulbs weight (g/pot) of Balady cv. was the highest among the cultivars (Table 3).

Table (3): Screening of garlic cultivars by using artificial soil with *P. terrestris*.

Garlic cultivar		Disease incidence (%)*, 100 days after planting	Yield (bulb weight; g/pot)
Balady	Healthy	00.00	39.6
	Infected	00.00	39.6
Seds-40	Healthy	00.00	48.2
	Infected	69.18	36.2
Chinese	Healthy	00.00	48.2
	Infected	69.18	36.2
American	Healthy	00.00	42.6
	Infected	58.30	34.2
Spanish	Healthy	00.00	30.4
	Infected	25.17	28.4
L.S.D. (5%)		3.32	2.91

*** symptoms of the pink root disease on garlic were apparent 90 days after planting in the infested soil. The pink root disease was measured on the basis of root pinking as described by Nicols *et al* (1965).**

Various biological methods have been used to determine resistance and susceptibility of garlic cultivars to pink root disease. Rating for pink root resistance were performed on garlic seedlings grown in artificial media amended with *P. terrestris* in addition to exposing garlic seedlings to Pyrenochaeta toxins in a separate test. These methods measured the effect of *P. terrestris* on the disease incidence but not on yield losses. Garlic seedlings were grown in pots amended with *Pyrenochaeta* inoculum in the greenhouse, this method was undertaken to determine pink root reaction of garlic cultivars and losses of yield, but symptoms of pink root on garlic started to appear 90 days after planting in the greenhouse. In this concern Thornton and Mohan (1996) found that symptoms of pink root is not usually apparent during early stages of onion growth when temperature are below optimum for growth of the pathogen. *P. terrestris* has the optimum temperature 28^{o}C (Shalaby *et al*., 2002). Absence of pink root late in the season may not adequately replicate the actual level of pink root resistance since resistance could be expressed as a voidance by early maturity before soil temperature became optimum for the pathogen, reduced susceptibility of roots to the pathogen or extensive replacement of infected roots with new healthy roots (Levy and Gornik , 1981).

A major advantage of using fungus-free toxins is that the problem with microbial contaminants would be greatly reduced (Hess and Weber, 1988). Hess (1966) demonstrated that the virulence of different isolats of *P. terrestris* varied significantly. Levy and Gornik (1981) and Netzer *et al* (1985) indicated that several weeks to months are required to screen onion cultivars for resistance to the onion pink root fungus *P. terrestris*, as well as soil treatment procedure takes time and is expensive (Katan *et al*, 1980). Therefore, the pathogen's toxins may be more useful in screening for a general resistance to garlic pink root disease, but do not measure yield losses.

Procedure using fungal toxins was the most reliable in the cultivar evaluation for resistance against pink root disease of garlic seedlings, in which, results could be obtained in only 7 days without serious problems from contaminants. To standardize seedling evaluation procedures with any of the method mentioned, more information will be needed concerning the presence of toxic components produced by the various isolates of the fungus *P. terrestris*.

In conclusion, biological assays using artificial media amended with *P. terrestris,* Pyrenochaeta toxins, or soil artificially infested with *P. terrestris* were developed to determine resistance of various garlic cultivars to the pink root disease. Biological assay using artificial medium amended with Pyrenochaeta toxins was the most reliable and time-effective for the evaluation of garlic cultivars for their resistance to pink root disease.

II. Physiological assays:

Histones content:

The leaves of cultivar Balady (resistant) werecharacterized by their significant high content of histones (poly lysine and poly arginine), 100 days

after planting whether the plants were healthy or infected, in comparison with Seds-40 ones (susceptible) (Table 4).

The results obtained are in line with those of Hadwiger *et al* (1977) who cleared that basic pea proteins rich in lysine and arginine are potentially more important in the pea tissue's resistance to plant pathogenic fungi. These histones inhibit the growth of *Fusarium solani* f. sp. *phaseoli* or *pisi in vitro*.

Ruiz-Carrillo *et al*. (1975) indicated that histones are synthesized in the cytoplasm and transported to the nucleus in synchronization with DNA synthesis within the cell. Once they have become localized in nucleoprotein, they may be unavailable to the fungus. However, Hadwiger *et al.* (1977) explained that changes in arrangement or number of potential for activation of histone genes could influence the potential for disease resistance. Shalaby *et al.* (2007) indicated that levels of histones, peroxidase and phenoloxidase activity, as well as RNA content were higher in pathogen-inoculated leaves of resistant garlic cultivar (Balady) than susceptible one (Seds-40).

It may be concluded that the resistant cultivar contained higher amounts of histones and therefore, the plant can resist *P. terrestris*. In other words, there is a positive correlation between histones content and resistance of garlic to *P. terrestris*.

RNA content:

It seems clear from the data presented in Table 4 that the infection with *P. terrestris* resulted in significant increase of RNA content in the leaves of resistant plants, 100 days after planting, as compared to the healthy ones. The leaves of susceptible cultivar (Seds-40) were characterized by their low content of RNA whether the plants were healthy or infected, as compared to the resistant cultivar (Balady).

Such finding may indicate that RNA plays a role in resistance of garlic plants to *P. terrestris* via their physiological role in different metabolic pathways.

Shalaby and Saeed (2000) indicated that the increase in RNA content of sesame plants may be a biochemical mechanism for induced systemic resistance in sesame plant against disease. Rasheed (2006) indicated that the infection by *P. terrestris* significantly increased DNA and RNA contents of leaves of both Seds-40 and Balady garlic cultivars, as compared with the healthy ones.

Free phenols content:

As shown in (Table 4), there were no insignificant differences between free phenols content of healthy and infected plants whether in resistance or susceptible cultivar. This mean that, free phenols of garlic plants did not constitute any significant importance in the physiology of resistance and susceptibility to Pyrenochaeta pink root disease. Although phenols play direct role in plant defence hence, Agrios (1988) indicated that several phenolic compounds or their oxidation products seem to induce resistance to disease through their inhibitory action on the pectolytic and other enzymes of the pathogen rather than on the pathogen itself and

apparently contribute to the resistance of the plant. However, Shalaby and Saeed (2000) indicated that free phenols of sesame plants dose not seem to be involved in induced resistance mechanisms by *Bacillus subtilis*, extract of *Helichrysum* flower, amino butyric acid, and potassium chloride against wilt disease.

Table (4): Effect of Pyrenochaeta pink root on histone, RNA, and free phenol contents* of resistant and susceptible garlic leaves (ug/gm fresh weight).

Garlic cultivar		Histones			RNA(*)	Free phenols
		Poly lysine	Poly arginine	Total		
Resistant (Balady)	Healthy	11.13	10.97	22	52.11	83
	Infected	13.12	18.88	32	63.22	83
Susceptible (Seds-40)	Healthy	10.10	9.90	20	17.07	81
	Infected	11.00	10.00	31	19.01	81
L.S.D. (5%)		1.39	1.68	3.15	8.01	N.s.

***These components were determined 100 days after planting.**

It is clear that physiological assay showed that histones and RNA contents in garlic plants play a role in resistance to the pink root disease.

REFERENCES

Agrios, G.N. 1988. Plant Pathology. Effect of pathogens on plant physiological functions. Academic Press, Inc. New York, 87-177.

Anonymous 1960. Official Methods of Analysis of the Association of Agricultural Chemists. Washington, D.C., USA, PP. 832.

Barnett, H.L. 1960. Illustrated Genera of Imperfect fungi. 2nd ed , Burgess Pub. Co., USA. PP:225.

Bonner, J.; Chalkley, G.R.; Dahmus, M.; Fambrough, D.; Fujimaura.; F.; Huang, R.C.; Huberman, J.; Hensom, R.; Marushige, K.; Ohlenbusch, H.; Olivera, B. and Widholm, J. 1968. Isolation and *characterization* of chromosomal nucleoproteins. Meth Enzymol. XII (B): 3-64.

Coleman, P. M. and Ellerbrock, L. A. 1997. Reachion of selected onion cultigens to pink root under field conditions in New York. Plant disease, 81 (2) 138-142.

Coleman, P. M.; Ellerbrochk, L.A. and lorbeer, J. W. 1992- Interaction of *Phoma terrestris* and soil moistur level on yield of two onion cultivars differentially susceptible to pink root. Plant Disease, 76-1213-1219.

Hadwiger, L.A.; Davis, C. L. and John, R. T. 1977. An evaluation uation of pea histones as disease resistance factores phytopatholoy, 67: 755-758.

Hess, W.M. 1966, Fixation and staining of fungus hyphae and host plant root tissues for electron microscopy. Stain Technol. 41: 27-35.

Hess. W. M. and weber, D. J. 1988. Assays for determining resistance and Susceptibility of onion cultivars to the pink root disease. Phytopathology. 78: 115-117.

Johns, E. W. 1964- studies on histones: Preparation methods for histone fractions From calf thymus Biochem. J. 92: 55-59.

Katan, J.; Rotem, I.; finnel, y., and Daniel, J. 1980, Solar heating of the soil for the control of pink root and other soil borne diseases in onions ipytoparasitica B: 39-50.

Kanvicka, o. 1984- Generative Reproduction von Knoblauch. Allium New Sl, 1: 28- 37.

Levy, D. and Gornik, A. 1981- Tolerance of onion to the pink root disease caused by *Pyrenochaeta terrestris*. Pyrenochaeta, 9: 51-57.

Netzer, D.; Rabinowitch, H.D. and Weintal, C. H. 1985. Greenhouse techninique to evaluate onion resistance pink root. Euphytica, 34: 385-391.

Nicols, C. G.; Gabelman, W. H.; Larson, R. H and Walker, J. C. 1965. The expression and inhentance of resistance to pink root in onion seedlings. Phytopathology, 55: 752-756.

Nitsan. J. and lang, A. 1966. DNA synthesis in the elongation nondividing cells of the lentil epicotyl and its promotion by gibberelin. Plant physiology, 41: 965-970.

Poter, I. J.; Merriman, P. R., and Keane, P.J. 1989. Integrated control of pink root of onions by dazomet and soil solarization. Aust. J. Agric. Res., 40: 861869.

Rasheed, A. A. 2006. Studies on Pink rot of garlic plants and its control under sandy soil conditions. Ph. D. Fac. Of Agric., Al- Azhar Univ., p: 137-138.

Rengwalska, M. M. and Siman, P.W. 1986- Laboratory evaluation of pink root and fusariun basal rot resistance in garlic Plant Disease, 70: 670-672.

Ruiz- Carrilo, A.; Wangh, L. J. and Allfrey, V.G. 1975, Processing of newly synthesized histone molecules. Science, 190: 117-128.

Sato, H.; Konoma, K. and *sakamura*,, S. 1981. Three new phytotoxins produced by *Pyrenochaeta terrestris:* Pyrenochaetic acids A. B and C. Agric Biol. Chem., 45: 1675-1679.

Shalaby, I. M. S. and Saeed. M.N. 2000. Biochemical defens mechanisms associated with the systemic induced resistance in sesame plants against Fusarium wilt disease. Zag. J. Agric. Res. 27 (1) 105-113.

Shalaby, I. M.S.; EL-Korashy, M. and Ismail, A.A. 2002. Pink root of garlic in Egypt: Occurrence, Pathogienicity and its relation with basal rot. Egypt. J. Appl. Sci., 17 (10) 544, 555.

Shalaby, I.M.S.; Yousef, R.M. and Rasheed, A.A. 2007: Evaluation of some garlic cultivars to infect with pink root disease caused by *Pyrenochaeta terrestris* and its relation to some biochemical changes. Zag J. Agric. Res. 34 (5) 871-880.

Sparace, S.A.; Judd, J.B.; Burke, B.B. and A asen, A.J. 1984: Pyrenocine C ophytotoxin – reltad metabolite produced by the onion pink root pathogen, *Pyrenochaeta terrestris*. Phytochemistry 23: 2693-3694.

Thornton, M.K. and Mohan, S.K. 1996. Response of sweet Spanish onion cultivars and numbered hybrids to basal rot and pink root. Plant Disease, 80: 660-663.

تقييم بعض الطرق التحليلية لتقدير مقاومة أصناف الثوم للإصابة بمرض الجذر القرنفلي

شلبي إبراهيم محمد شلبي و صابر محمد على مرسى و أحمد على عبد الباقى.
معهد بحوث أمراض النباتات- مركز البحوث الزراعية – الجيزة

أجرى هذا البحث بغرض معرفة أفضل الطرق البيولوجية والفسيولوجية في تقدير درجة المقاومة في أصناف الثوم للإصابة بمرض الجذر القرنفلي المتسبب عن الفطر بيرينوكيتا ترستس. وأوضحت النتائج اختلاف الطرق البيولوجية والفسيولوجية في تقدير مدى مقاومة أصناف الثوم لمرض الجذر القرنفلي. وقد كانت أكثر الطرق البيولوجية فعالية هي طريقة استخدام توكسينات الفطر بيرينوكيتا ترستس في تقدير حساسية أصناف الثوم للإصابة بالمرض وقد تبين من الطرق الفسيولوجية فعالية الهسنونات والحمض النووي ريبونيوكليك RNA في مقاومة نباتات الثوم للإصابة بمرض الجذر القرنفلي.

قام بتحكيم البحث

أ.د / ياسر محمد نور الدين شبانه	**كلية الزراعة – جامعة المنصورة**
أ.د / احمد السيد الكورانى	**كلية الزراعة بدمنهور – جامعة البحيره**