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Study on the control of *Fusarium* wilt in the stems of mycorrhizal and trichodermal inoculated pepper (*Capsicum annum* L.)

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ABSTRACT:
Objective: Green house experiments were carried out to investigate the effect of arbuscular mycorrhiza (AM) (*Glomus mosseae*) and *Trichoderma koningii* as biocontrol agents against *Fusarium* wilt of pepper (*Capsicum annum* L.) caused by *Fusarium oxysporum* f.sp lycopersici.
Methodology and results: The pepper used was susceptible to the pathogen (*F. oxysporum*). The pepper plants inoculated first with *F. oxysporum* died after 2 to 3 weeks. Simultaneous inoculation with the AM and the pathogen exhibited improved growth performance over the arbuscular mycorrhiza fungus non-inoculated. AM inoculation a week before *F. oxysporum* inoculation had the best growth performance. However, simultaneous inoculation of the pathogen and *T. koningii* did not improve the growth of the pepper. Microscopic examination of cells showed that the *Glomus mosseae* and *T. koningii* inoculated plants had cell walls that were thickened at the edges compared to those from other treatments.
Conclusion and application of findings: The inoculations of pepper with these biocontrol agents reduced the incidence of *Fusarium* wilt in it and this depends on the sequence of AM inoculation. The Fusarium wilt of pepper could be biologically controlled by *G. mosseae* and *T. koningii* inoculations.
Key words: biocontrol, defense mechanism, *F. oxysporum, G. mosseae*, growth performance, pathogen, pepper, *T. koningii*.

INTRODUCTION

Disease problems and control are by no means peculiar to modern agriculture. Farmers are in general familiar with chemical pesticides because of their quick, effective actions. However, chemical control poses risks to human lives and environments hence the necessity of changing to biological control (Denis, 1971). The use of chemical pesticides increases the cost of production and also makes the products more expensive (FAO, 1989). Another remarkable feature of chemical pesticides is the capacity of the pests and disease species to quickly evolve genetic resistance to the plethora of chemical agents used against them (Dean, 1991). Soil erosion and underground water contamination have been known to be caused by pesticide residues and fertilizer runoffs (Basim *et al*., 1999).
As environmental and health hazards mount as a result of heavy pesticide usage, , a new holistic perspective emerges in food production - sustainable agriculture and this is a dynamically evolving system in which widely divergent agricultural practices and conditions are evaluated, modified and verified in order to create a productive and continuing sustainable agriculture (Salami *et al*., 2005). This concept is the biological or better still crop protection. It is environmentally oriented ,is easy to use and affordable (IBPGR, 1992). Biological control of pathogens has proven not only ecologically but also economically sound

as seen in the benefit/cost ratio (Salami *et al*., 2005; Kiss, 1991).

Pepper has become an important crop in the tropics and is needed in many households to enhance or improve intake of balanced diet. It also serves as good sources of income to the resource-poor farmers in many developing countries (Oyetunji and Osonubi, 2005). However, its production is been hampered by many diseases one of which is *Fusarium* wilt caused by *Fusarium oxysporum* that causes vascular wilt of many crops (Kraft and Papavizas, 1983; Kucuk and Kivanc, 2003; Siddiqui and Akhtar, 2007).

Arbuscular mycorrhizae are ubiquitous symbiotic associations that are important to normal plant growth of most crops and as such tend to optimize crop production (Reid, 1990; Oyetunji and Osonubi 2008). They are agents of both plant and soil nutrition (Smith and Read, 1997).

Formation of arbuscular mycorrhiza brings about changes in root morphology and significant changes in root physiology. AM fungi are known to reduce soil borne diseases or the effects of disease caused by fungal pathogens (Dehne, 1982). The method of control is basically antagonism of these pathogens. Suppression of the diseases by AM fungi is also attributed to increased nutrient uptake (particularly phosphorus) by mychorrhizal plants (Linderman, 2000).

Trichoderma are also common soil inhabitants. They are major mycoparasites which parasitize a large number of plant pathogens (Liu and Baker, 1980). They also produce antibiotics. They therefore control many soil borne diseases such as damp off (Hulang 1980). There is little information on the comparison of Trichoderma and AM fungi in control of Fusarium wilt of pepper. The simultaneous and spatial inoculations of these two antagonists have not been investigated.

The objectives of this study were to investigate the efficiency of simultaneous and spatial inoculations of *T. koningii* and *G .mosseae* in the control of Fusarium wilt in pepper and their effects on the cell structures of the infected plants. Dandurand and Knudsen in, 1993 reported that individual biocontrol agent may not sufficiently control wide spectrum of pathogens, and that development of compatible combinations of control agents is a promising research direction.

MATERIALS AND METHODS

This investigation was carried out at the Department of Botany and Microbiology, University of Ibadan, Nigeria. The Fusarium susceptible bell pepper (*Caspicum annum*) variety NHU-2C was obtained from National Horticultural Research Institute, Ibadan. While the *Trichoderma koningii* and *Fusarium oxysporum* f.sp. lycopersici were collected from International Institute of Tropical Agriculture (IITA) Ibadan. The *G. mosseae* (Nicholson and Gerdermann) Gerdermann and Trappe was supplied by the Department of Botany, University of Ibadan, Nigeria. The pepper seeds were surface sterilized in 10% NaOCl for five minutes. The seeds were raised in the nursery in a sterilized soil and were transplanted after two weeks to the nursery polyethylene bags already filled with sterilized soil to the brim and watered to 60% field capacity. The cultures of *T. koningii* and *F. oxysporum* were sub-cultured in a PDA medium and their suspensions were made with sterile water.

Experimental design: The treatments were laid out in a completely randomized design. The treatments were mycorrhizal, *Trichoderma* and *Fusarium* inoculations. There were two levels of each treatment (i.e. simultaneous and spatial inoculations). These were replicated six times. The experiment was repeated three times and the average calculated were applicable.

Inoculations: The Trichoderma and *Fuasrium* inocula were obtained from the fresh growth of these organisms on PDA. Their suspensions were obtained by washing their 6day old media into different 100ml of distilled water. The Trichoderma suspension consisted of the hyphae and their conidia, while *Fusarium* suspension contained the hyphae, macro and microconidia. These suspensions were used as the inocula. The number of conidia in the suspensions was estimated to be to be very high. The pepper seedlings were carefully uprooted and had their roots rinsed thoroughly in sterile water to remove the attached soil particles. The roots of the seedlings for simultaneous inoculations were dipped into the suspension of *F. oxysporum* and *G. mosseae*. The suspension consisted

of 5 ml of the former and 450 spores per 100 g of soil of the latter. The suspension was poured into the dug hole where the seedlings were planted.. This procedure was also employed for the simultaneous inoculation of *T. koningii* and *F. oxysporum*. The Spatial inoculations were carried out by separate inoculations of the fungi at different times.

a). i. *F. oxysporum* was inoculated first and a week later *G. mosseae* was inoculated. ii. *G. mosseae* was first inoculated and a week later *F. oxysporum* was inoculated.

b). these procedures were also adopted for *T. koningi* and *F. oxysporum* spatial inoculations.

Growth Parameters: Certain growth parameters such as plant height, girth, number of leaves produced and total leaf areas were monitored weekly. The measurement of these parameters started at four weeks after transplant (WAT). *Fusarium* wilt symptoms observed were leaf chlorosis and dropping, leaf shedding from the base, hypertrophy at the base, stem rot at the base and later plant death.

Sectioning: The pepper plants were carefully uprooted at the end of 11th week and washed in tap water. The roots were then cut into bits with razor blades and stored in 50% ethanol for further studies. Sledge (sliding) microtone was used for sectioning. The thickness was set at 30µm. The sections were preserved in 50% ethanol again.

Staining: Saffranin and Lactophenol blue were used. Good sections from each replicate were selected and washed in tap water and were then transferred into saffranin and allowed to stay for 3 min. Thereafter they were washed again in tap water and transferred into Lactophenol blue where they stayed for another 3 min for counter staining. They were later removed and washed again in tap water. The good stained sections from each replicate were mounted in 25% glycerol and observed under high power microscope and photographs taken.

Estimation of mycorrhizal root colonization: The percentage mycorrhizal colonization of the fibrous roots of the plants were determined by collecting randomly fine root segments (about 2g) from each harvested plant within each plot. They were stored in 50% ethanol until further analyses. The fibrous roots were later cleared in 10% KOH solution for 20min at 121^0C and further bleached in alkaline H_20_2 before rinsing thoroughly in running water. The bleached roots were soaked in 1% HCl for 3min after which the roots were stained in 0.05% Trypan blue in 500ml of glycerol, 450ml of water and 50ml of HCl. The percentage root infection was then determined by grid line intersects method of Giovannetti and Mosse, (1980).

Statistical analysis: The data were subjected to ANOVA using SAS package (SAS Institute 1996) and t-tests were performed to separate the means of the measured parameters

RESULTS

Mycorrhizal root colonization*:* The roots of *G. mosseae* treated pepper were colonized by the AM fungus. Others were not. The percentage colonization was significantly lower in F-M treatment. The rates of colonization were high (above 59%) in the other *G. mosseae* treated pepper.

Growth parameters: Significant differences ($P \leq 0.05$) were recorded in some of the growth parameters measured in all the treatments (Table 1, Figs. 1, 2, 3 and 4).

Table 1: The effects of mycorrhizal and Trichodermal sequence on the growth of *Fusarium* infected pepper at harvest 11th week after transplanting

Treatment	% Mycorrhiza Colonization	Plant Height (cm)	Stem Girth (cm)	Biomass (g plant^{-1})	Leaf Number	Total Leaf Area (cm^2)
T	0	19.5^a	2.2^a	30.9^c	11^a	82.2^b
T-F	0	15.58^b	1.6^a	32.5^c	9^a	85.0^b
TF	0	16.2a^b	1.88^a	40.7^{bc}	11^a	98.5^b
F-T	0	0	0	0	0	0
C	0	20.5^{ab}	2.4^a	51.6^b	11^a	128.6^a
M	67.2^b	22.0^a	2.03^a	55.9^{ab}	11^a	130.0^a
F	0	0	0	0	0	0
M-F	71.9^{ab}	22.4^a	2.6^a	67.5^a	13^a	148.6^a
F-M	49.1^c	0	0	0	0	0
MF	82.7^a	22.0^a	2.8^a	61.7^{ab}	13^a	140.0^a

The data were all transformed. The values with same letters were not significantly different at $P \leq 0.05$ and were the means of the three experiments. The means were separated with t-test.
T = Inoculation with *T. koningii*; T-F = Spatial inoculation with *T. koningii* and *F. oxysporum*
TF = Simultaneous inoculation with *T. koningii* and *F. oxysporum;* M = *G. mosseae* inoculation; F-T = Spatial inoculation with *F. oxysporum* and *T. koningii*; F = inoculation with *F. oxysporum;* M-F = Spatial inoculation with *G. mosseae* and *F. oxysporum*; F-M = Spatial inoculation with *F. oxysporum* and *G. mosseae*; MF = Simultaneous inoculation with *G. mosseae* and *F. oxysporum*

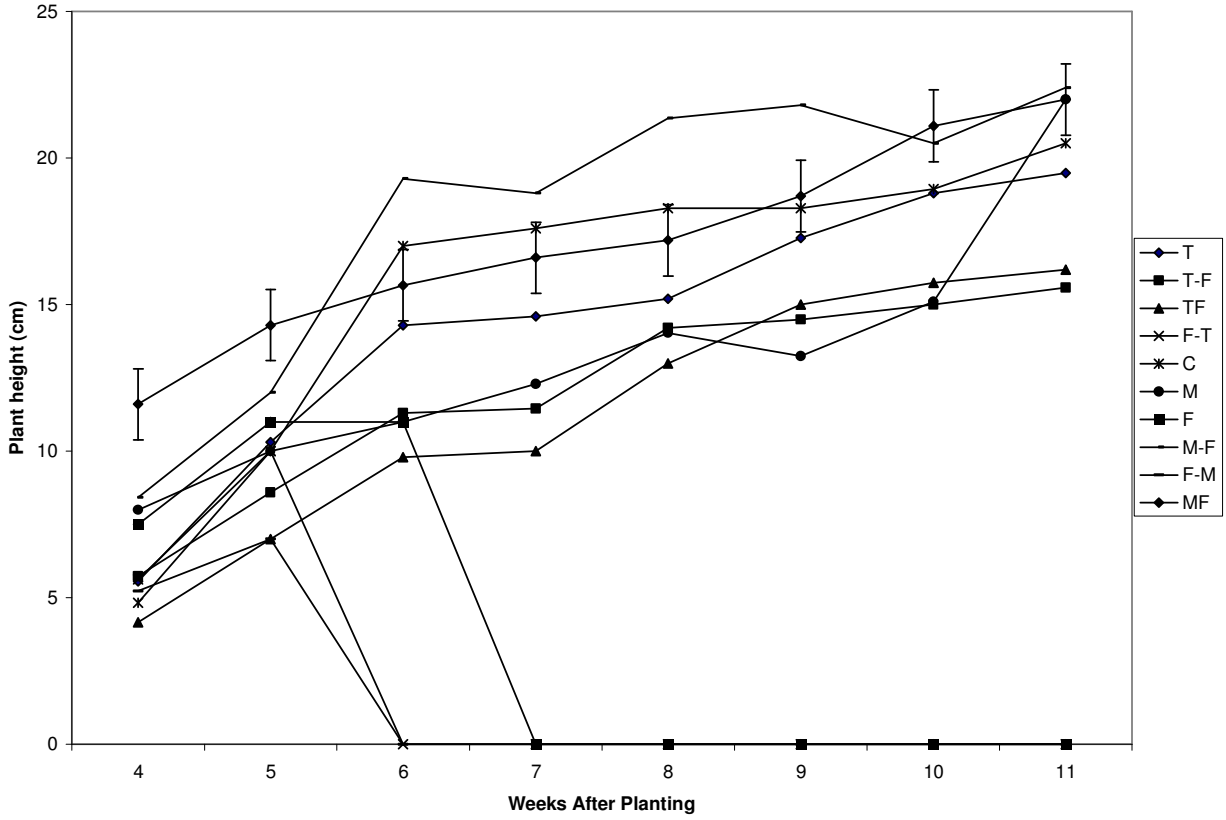

Figure 1: Plant heights showing the effects of the pathogen and the antagonists
The values were the means of the three experiments. The error bars represent the standard error.
T = Inoculation with *T. koningii*; T-F = Spatial inoculation with *T. koningii* and *F. oxysporum* ,TF = Simultaneous inoculation with *T. koningii* and *F. oxysporum;* M = *G. mosseae* inoculation; F-T = Spatial inoculation with *F. oxysporum* and *T koningii*; F = inoculation with *F. oxysporum;* M-F = Spatial inoculation with *G. mosseae* and *F. oxysporum*; F-M = Spatial inoculation with *F. oxysporum* and *G. mosseae*; MF = Simultaneous inoculation with *G. mosseae* and *F. oxysporum*.

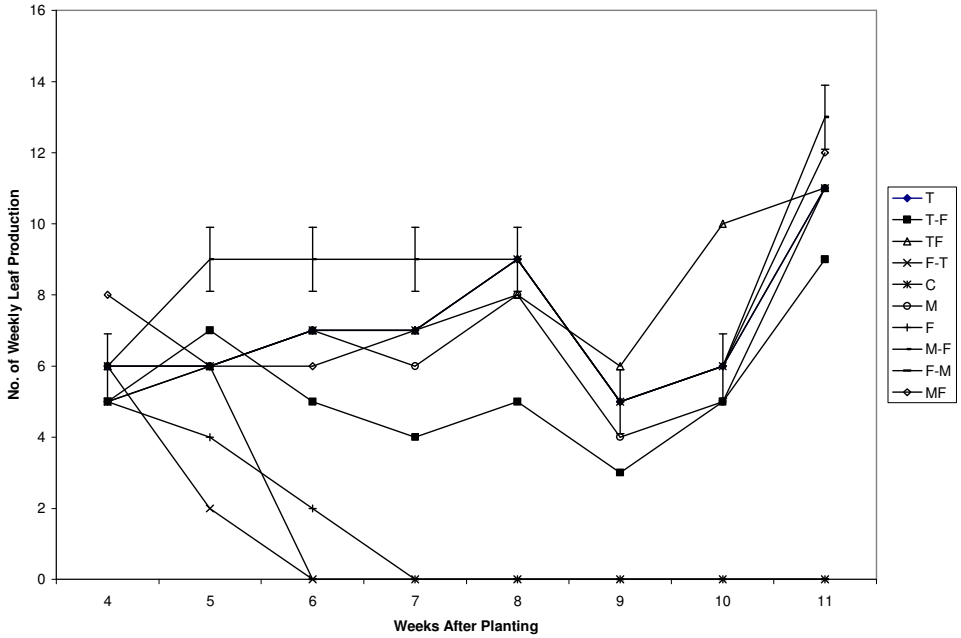

Fig. 2: Effects of the pathogen and the antagonists on the weekly number of leaves production
The values were the means of the three experiments. The error bars represent the standard error.
T = Inoculation with *T koningii*; T-F = Spatial inoculation with *T. koningii* and *F. oxysporum* ,TF = Simultaneous inoculation with *T. koningii* and *F. oxysporum;* M = *G. mosseae* inoculation; F-T = Spatial inoculation with *F. oxysporum* and *T. koningii*; F = inoculation with *F. oxysporum;* M-F = Spatial inoculation with *G. mosseae* and *F. oxysporum*; F-M = Spatial inoculation with *F. oxysporum* and *G. mosseae*; MF = Simultaneous inoculation with *G. mosseae* and *F. oxysporum*.

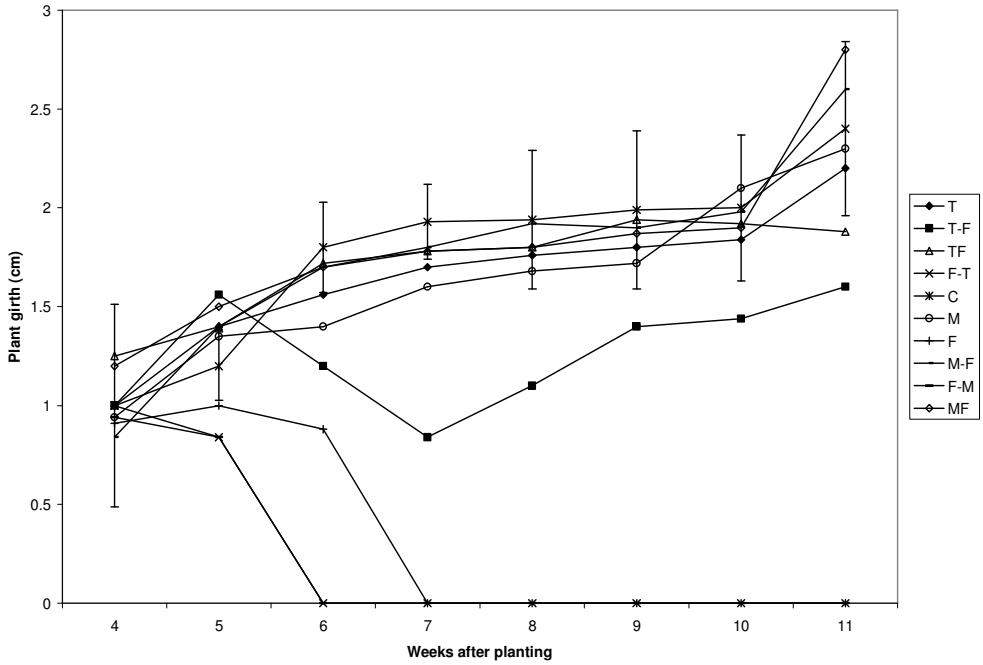

Figure.3: The effects of the pathogen and the antagonists on the pepper girth
The values were the means of the three experiments. The error bars represent the standard error.
T = Inoculation with *T. koningii*; T-F = Spatial inoculation with *T. koningii* and *F. oxysporum* ,TF = Simultaneous inoculation with *T. koningii* and *F. oxysporum;* M = *G. mosseae* inoculation; F-T = Spatial inoculation with *F. oxysporum* and *T. koningii*; F =

inoculation with *F. oxysporum;* M-F = Spatial inoculation with *G. mosseae* and *F. oxysporum*; F-M = Spatial inoculation with *F. oxysporum* and *G. mosseae*; MF = Simultaneous inoculation with *G. mosseae* and *F. oxysporum*.

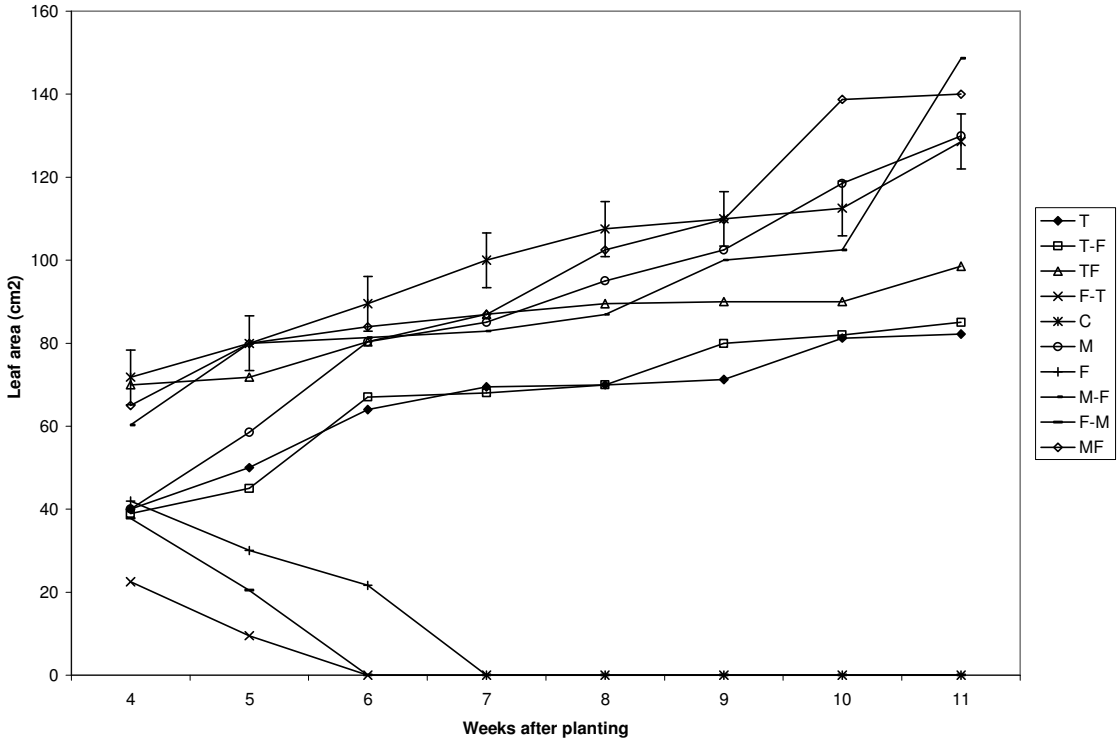

Figure 4: The effects of the pathogen and the antagonists on the surface leaf area of pepper
The values were the means of the three experiments. The error bars represent the standard error.
T = Inoculation with *T. koningii*; T-F = Spatial inoculation with *T. koningii* and *F. oxysporum* ,TF = Simultaneous inoculation with *T. koningii* and *F. oxysporum;* M = *G. mosseae* inoculation; F-T = Spatial inoculation with *F. oxysporum* and *T. koningii*; F = inoculation with *F. oxysporum;* M-F = Spatial inoculation with *G. mosseae* and *F. oxysporum*; F-M = Spatial inoculation with *F. oxysporum* and *G. mosseae*; MF = Simultaneous inoculation with *G. mosseae* and *F. oxysporum*.

Those plants inoculated alone with *F. oxysporum* (without any of the antagonists) could not survive beyond the third week of transplanting. The plants with simultaneous, spatial and single inoculations of the AMF survived and had better growths than those of the other treatments. Those treated with *T. koningii* also survived except those under spatial inoculation (where the pathogen was introduced one week before the *Trichoderma* was introduced (Table 1, Figs. 1, 2, 3 and 4). Simultaneously and spatially inoculated pepper with both the AMF and the pathogen had the best plant heights. The AMF singly inoculated pepper performed below those of M-F, C, MF and T inoculated at the initial stage, but caught up with them at week 10 after transplanting.

The controlled plants showed consistent increase in plant girth with time, although the plants inoculated simultaneously with the AMF and the pathogen had the overall best girth, followed by the plants inoculated singly by the AMF and then those spatially inoculated with the AMF and the pathogen (Fig 3). The girth of the spatial inoculation between *T. koningii* and the pathogen (i.e. where the former was first inoculated) was the least among the plants treated with the antagonists. It was reduced at week 5 after transplanting (ATP), but picked up after 8th week after transplanting. The leaf surface area of the plants inoculated first with the AMF before the pathogen (spatial inoculation) was the highest and was increasing with time. However, the leaf surface area of the spatial inoculation between the *Trichoderma* and the pathogen was decreasing with time at the earlier stage before it started increasing again at week 9. The leaf areas of *Trichoderma* treated peppers were the least among the plants treated with antagonists (Fig. 4).

The final result at harvest revealed similar trend (Table 1). The highest leaf area was obtained in the M-F, MF, M and C in that order, though there was no significant

different between them at P ≤ 0.05. The leaf areas of the Trichoderma treated pepper were significant lower than those of mycorrhizal treated and control plants. The biomass obtained followed a similar trend.

Microscopic studies: Microscopic studies revealed that the cells of the pepper plant inoculated with *F. oxysporum* were degraded, while those of the control plants were intact (not degraded). The cells of the plants inoculated with the AM fungus were found to be highly thickened at the edges. This was similar to what was observed in those cells of the plants inoculated with *T. koningii.* The thickenings were more pronounced in the simultaneous and spatial inoculations than when the antagonists were inoculated alone (without the pathogen) (Plates: 1, 2, and 3).

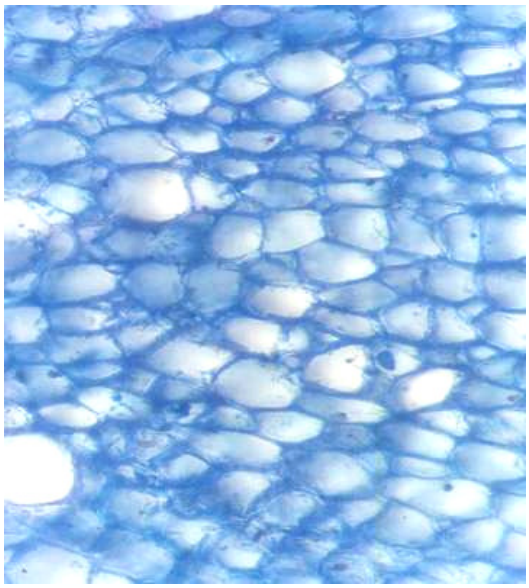

Plate 1: Enlarged intact cells of the control plants

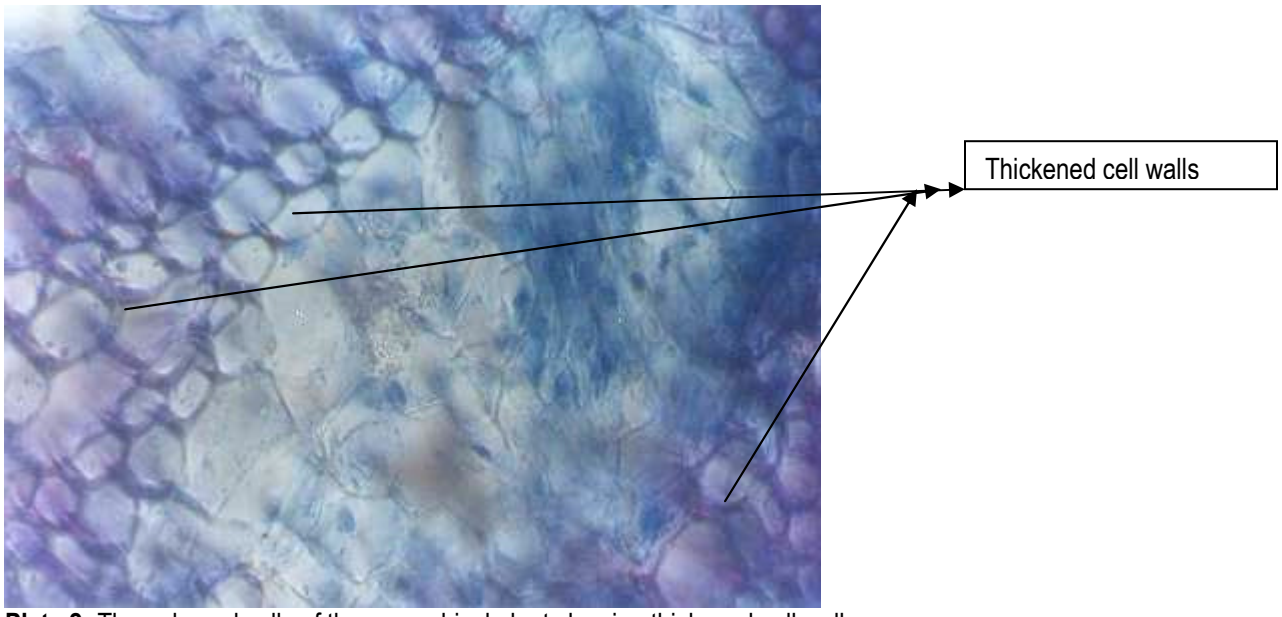

Plate 2: The enlarged cells of the mycorrhizal plant showing thickened cell walls

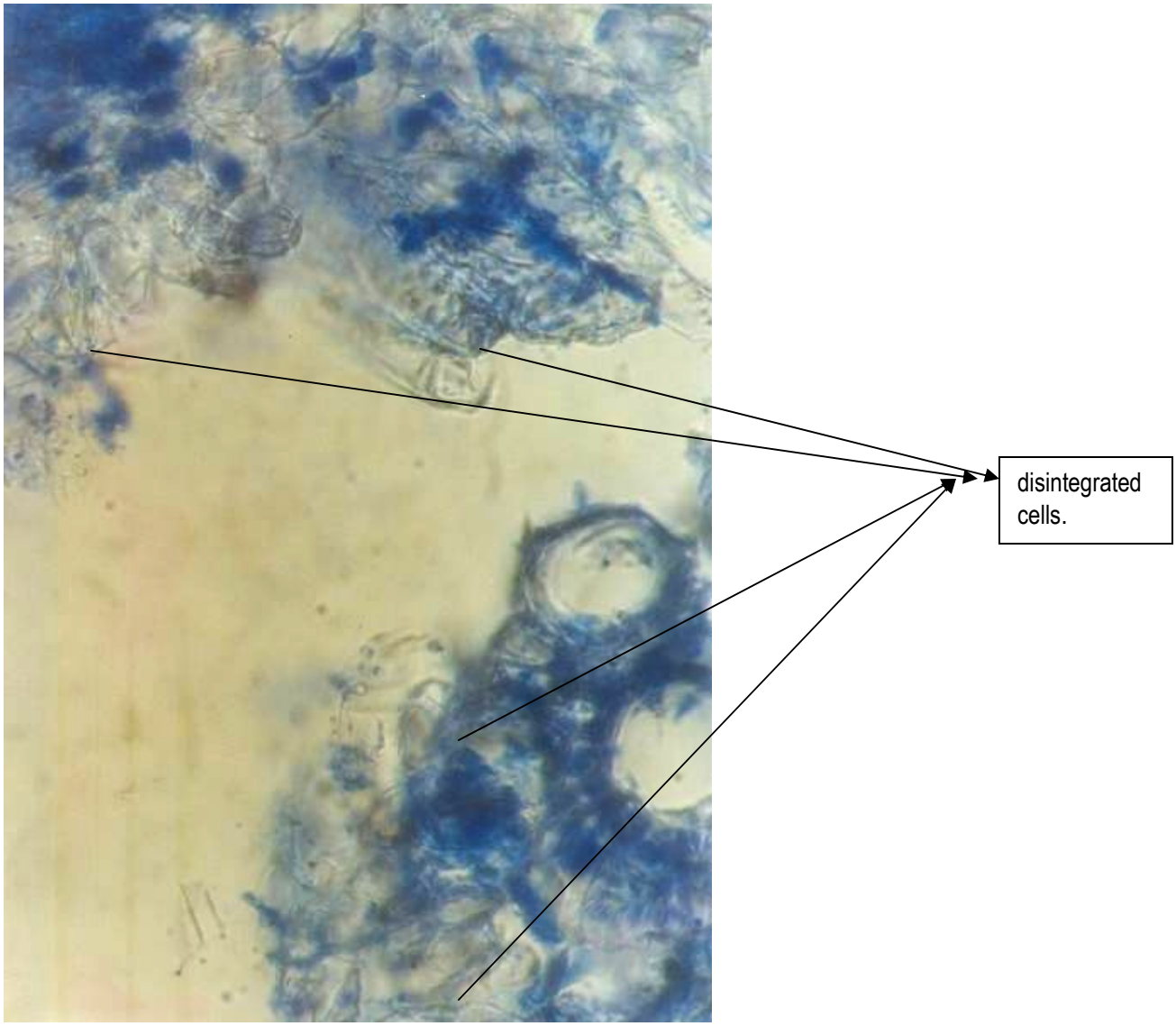

Plate 3: Degraded (disintegrated) cells of *F. oxysporum* infected pepper plant

DISCUSSION

The symptoms of Fusarium wilt observed on the pepper plants were similar to those earlier described by Garcia (1933). Those plants inoculated with *F. oxysporum* only and those inoculated spatially with the pathogen and *T. koningii* died after two weeks of inoculation. This confirms the findings of Garcia (1933) that complete pepper wilting occurs between 2 weeks to 3 months after attack. However, this depends on the prevailing condition. Kucuk and Kivanc, 2003 reported that *F. oxysporum* was resistant to *T. harzianum*.

There was no stem rot or decay in *T. koningii* inoculated plants as was the case with *F. oxysporum* inoculated plants. The growth pattern of those plants inoculated with *T. koningii* was far below those inoculated with *G. mosseae*. While those inoculated with the later were growing steadily, those of the former were hindered at the early stage before they improved at the later stage of the experiments. This clearly showed the difference in the benefits both antagonists conferred on plants. The benefits of the mycorrhiza outweighed that of the *Trichoderma*. The result shows that there was no mycorrhizal colonization in control, *Fusarium*, and *Trichoderma* treatments.

The plants inoculated with the pathogen died between 2 and 3 weeks of inoculation. However, those plants simultaneously inoculated survived. Spatial inoculation could not ameliorate the symptoms of the pathogen when it was inoculated a week before the *T. viride*.

However, synergetic effect was observed in the pepper plants inoculated spatially with the AMF and the pathogen. Plants inoculated with the AMF had improved growth which supports the findings of earlier workers (Amusat *et al*., 2008; Linderman, 2000). None of the AMF inoculated plants died. This might be due to increase in nutrient uptakes which conferred vigour to the pathogen infected plants. Other factors such as stimulation of growth hormones production by the AMF could be responsible. Inhibition of germination of the pathogen's spores might be another factor. Antagonistic organisms applied to seeds prior to planting colonize the rhizosphere of seedlings and thus are present at or near the pathogens "infection court" where they act by producing antifungal compounds through hyper parasitism (Dandurand and Knudsen, 1993). Ability of the AMF colonized plants to withstand transplant shock and water stress is also another reason why those plants grew better. The spatially inoculated pepper with the AM fungus developed very well than those inoculated with AM fungus only, so also those inoculated simultaneously with the AMF and the pathogen. This might be due to the ability of the plant to utilize the pathogen's carbohydrates after the pathogens had been probably degraded by the AMF enzymes. This has not been reported in the previous works.

The inhibition of the pathogenic infection was as a result of lignifications of the AMF colonized roots cell wall and wound barrier formation as found in the highly thickened cells of those plants colonized by the mycorrhiza. These caused reduction in *Fusarium* wilt symptoms. This was supported by the findings of Dehne, 1982.

The plants inoculated with *T. koningii* showed differences in height. Those plants inoculated with *T. koningii* alone later out performed the control plants while those simultaneously inoculated with *T. koningii* and the pathogens performed below those of control, though there were no symptoms recorded. This finding was similar to those of Liu *et al*. 1980 and Chet *et al*. 1981.

One of the modes of inhibition of the pathogen by the two antagonists was found to be the inability of the pathogen to degrade the cells of the plants inoculated with the antagonists. While the cells of those plants inoculated alone with the pathogen were degraded and those inoculated with the antagonists were found to be intact. The cell walls of the plants inoculated with the *G. mosseae* were much thickened particularly at the edges. This was not found in the plants inoculated singly with either of the two antagonists. This suggests the thickening was mediated by the presence of the pathogen. It was evident from this study that biocontrol of *Fusarium* wilt in pepper is visible.

The Fusarium wilt of pepper could be biologically controlled by *G. mosseae* and *T. koningii* inoculations. However, the antagonists must be inoculated at least a week before the attack by the pathogen. Preferably, the antagonists should be incorporated into the nursery beds before sowing the pepper seeds or the seeds could be coated with these antagonists for effective control.

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