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CONTENTS

ANALYSIS OF THE CHARACTERS ON SOME ANGELICA TAXA

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Angelica archangelica was at times confused with *A. sylvestris* subsp. *montana*, especially for size, vesicular vaginas and spherical umbels. The essential difference between them is reflected by the fruit not attached to the seed, whitish, with numerous thin secretory channels, not visible from the outside at *A. archangelica* and connate to the seed, brown, with six large secretory channels, externally visible as black stripes on the *montana* subspecies.

Key words: plant character analysis, fruits, *Angelica*, Romania.

INTRODUCTION

The representatives of Apiaceae family could be separated mainly by the morphology of mature fruit (Figs. 1, 2). In their absence, identification only by vegetative organs is uncertain, often leading to confusion. This kind of situation is considered to have occurred to the species of the genus *Angelica* from the flora of Romania. Specimens from the Retezat Mountains resembling, at first sight, in habit *A. archangelica* L. were collected periodically but following analysis of the fruit, they were found to be different. This is the reason that led us to examine the *Angelica* species closer, with the following main question: is *A. archangelica* present in the spontaneous flora of Romania? J. Cannon (1968) states it is not. E. Yankova and Z. Cherneva (2007) recently declare the same for Bulgaria.

History. Starting from F. Schur (1866) it arises that five *Angelica* species grew in Transylvania: *A. sylvestris* L., *A. alpina* Schur, *A. macrophylla* Schur, *A. montana* Schleich. and *A. archangelica* L. D. Brandza (1879–1883) mentions *A. sylvestris* L., including var. *elatior* Wahlenb. (Predeal, Olăneşti, Horez) and *A. archangelica* L. (Dobrovățului Floodplain near Iassy and Peştera Cave from the Bucegi Mountains). L. Simonkai (1886) restricted the species indicated by Schur to three: *A. sylvestris* L. (= *A. archangelica* Baumg non L., *A. pratensis* Baumg. non M. Bieb. and *A. macrophylla* Schur), *A. montana* Schleich. (= *A. alpina* Schur) and *Archangelica alpina* Wahlenb. (*Archangelica officinalis* Hoffm. p. p.). D. Grecescu (1898) quotes *A. sylvestris* L. var. *elatior* Wahlenb. and *Archangelica officinalis* Hoffm. (= *Angelica archangelica* L., *A. archangelica* var. *alpina* Wahlenb.) from

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Bucegi, just like D. Brandza. A. Borza (1949) mentions three species: *A. sylvestris* L., and var. *elatior* Wahlenb. (= *A. montana* Schleich.), *A. archangelica* L. (*A. officinalis* Hoffm., *A. sativa* Besser) and rarely *A. palustris* (Besser) Hoffm. This latter classification is present both in I. Todor (1958) and A. Beldie (1972, 1977) and in V. Ciocârlan (2000).

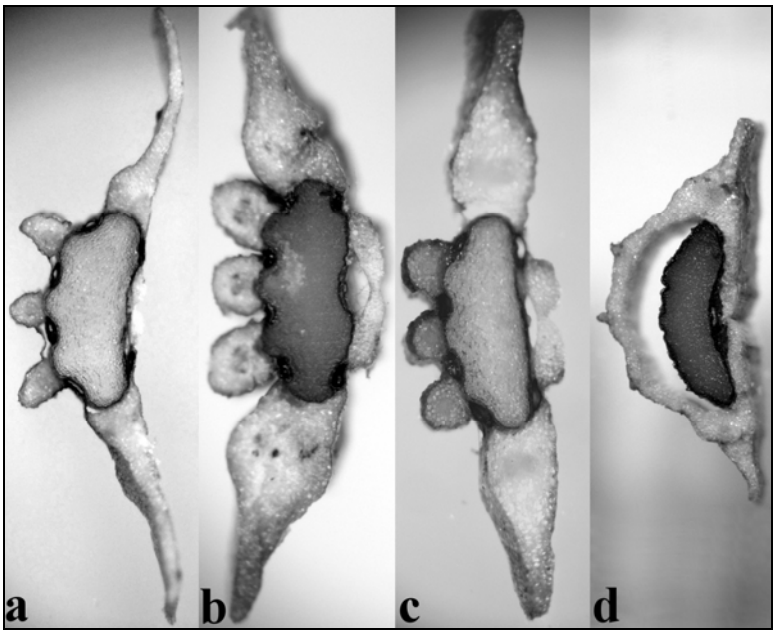

Fig. 1. *Angelica* mericarps cross-section: a – *Angelica sylvestris*; b – *Angelica sylvestris* subsp. *montana*; c – *Angelica pancicii*; d – *Angelica archangelica*.

Fig. 2. *Angelica* fruits: **a** – *Angelica sylvestris*; **b** – *Angelica sylvestris subsp. montana*; **c –** *Angelica pancicii*; **d** – *Angelica archangelica*.

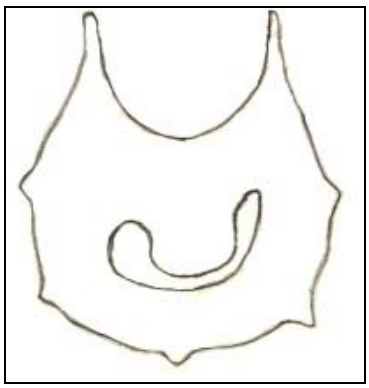

Fig. 3. *Angelica sylvestris* subsp. *sylvestris* – petiole cross-section.

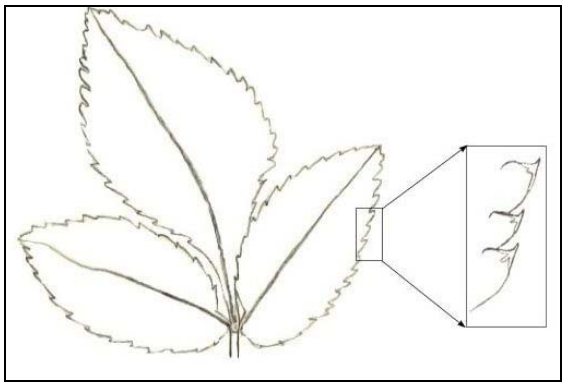

Fig. 4. *Angelica sylvestris* subsp. *sylvestris* – terminal leaflets with toothed margin (in detail).

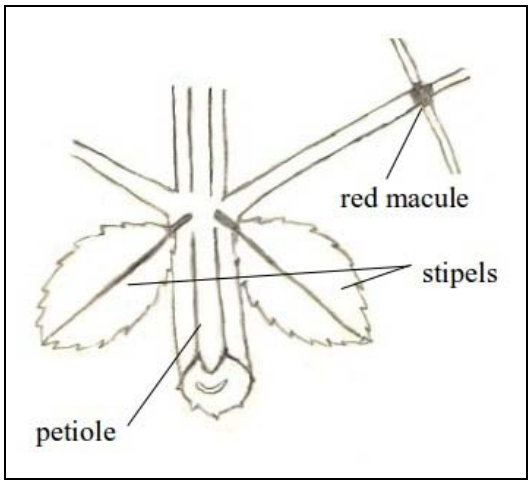

Fig. 5. *Angelica sylvestris* subsp. *sylvestris* – petiole cross-section and stipels.

Fig. 6. *Angelica sylvestris* subsp. *montana* inflorescences, Râul Mare – the Retezat Mountains.

Fig. 7. *Angelica sylvestris* subsp. *montana* – long decurrent and connate subterminal leaflets.

Fig. 8. *Angelica archangelica* herbarium specimen collected by E.I. Nyarady in 1904 from the Retezat Mountains (BUCA Herbarium). In reality, it is *Angelica sylvestris* subsp. *montana*.

E. Nyárády (1958) quotes from the Retezat *A. archangelica*, *A. sylvestris* var. *elatior* Wahlenb. (*A. montana* Schleich.) and describes the hybrid *A.* × *mixta* Nyár. ex Todor (*A. sylvestris* × *A. archangelica*), but only by vegetative organs (without any reference to fruit!), making it impossible to tell which species he refers to.

MATERIAL AND METHODS

Because the examination of vegetative organs did not lead to the real identification of specimens collected from the Retezat Massif (Lăpuşnicului Valley, Râu Bărbat Valley) and Sebeşului Valley, we carefully studied the fruit (color, size, cross section, ratio between fruit and seed) that proved to be the organ with stable characters.

As information sources we used classic botanical literature (*Flora Europaea*, *Flora of Romania*, *Flora of Bulgaria*, etc.), but also papers relating to *Angelica* (Yankova, Cherneva, 2007). Various plant characters were investigated in live and illustrated material.

DISCUSSION AND RESULTS

First, we should emphasize that the herbarium material is almost always inappropriate for taxonomic research. Either there are only fragments, due to the large size of the plants, often missing the lower leaves and lower stem leaves or the mature fruits that yield safe identification, or the fruits are from the lateral umbels that are easier to press and not from the central, more enlightening, umbel.

Angelica species separation has been achieved in our literature by the shape of inflorescence, leaflets decurrence, vaginas size and the cross section shape of the lower leaves and lower stem leaves (Tikhomirov *et al*., 1996). Only one author (Todor, 1958) uses a special character of the fruit, which we found in a book published over a hundred years ago (Schinz, Keller 1900). This character, the ratio between pericarp and seed, stopped us from attributing material from Retezat to *A. archangelica* and made us believe that it is a form of *A. sylvestris*, converged with the previous species, with robust stature, powerful vesicular vaginas, cylindrical petiole, globular umbel (Fig. 6), decurrent upper leaflets (Fig. 7), or *A. mixta.* The discordant organ is the fruit, which resembles well the one from *A. sylvestris*, but some plant characteristics do not match those of typical *A. sylvestris*. We must mention that there are some specimens of *A. sylvestris* whose mericarps have very narrow lateral wings, like those of *A. archangelica*, separate in subtaxon fo. stenoptera (Boiss.) Thell.

Now we ask the same question: is *A. archangelica* present or not in the spontaneous flora of Romania? To answer this, we consulted *The Herbarium* of

The Faculty of Agronomy in Bucharest, where we found material identified by I. Todor, the monograph of *Angelica* genus in the *Flora of Romania*.

Influenced by some authors (Cannon, 1968), we initially considered the specimens from the Apuseni Mountains (Someşului Rece Valley, between Power Plant and Răcătău District, leg. I. Todor 1949) as being escaped from cultivation, but when we found material from the Bucegi Mountains (Ialomiței Valley), the Făgăraş Mountains (at the base of Râiosu Mountain) and from the Retezat Mountains (Pietrele Valley and Stânişoarei Valley), the answer was clear: *A. archangelica* grows spontaneously in our flora! The authors found the species in Râu Bărbat Valley, at 1400 m altitude.

In literature, there are different keys for the separation of taxa. If we use an eastern European separation key for the two species (Voroshilov *et al*., 1966), we can state that the fruit material collected from Retezat resembles more that of *A. sylvestris*:

1. flowers white or slightly pink; umbels and umbellules non-spheric, fruits brown, with winged wide lateral ribs – ***A. sylvestris***;
1. flowers yellow-green; umbels and umbellules spheric, fruits pale yellow or almost white, with narrow lateral ribs – ***A. archangelica***.

Another key (Rothmaler, 1966) begins, more appropriately, with the color of the plant:

1. plant dark green, petiole and midrib adaxial channelled, peduncle hairy on the entire length, corolla white or reddish, almost green before flowering – ***A. sylvestris***;
1. plant pale green, petiole cylindric, midrib channelled; penduncle hairy only on the apex, corolla greenish – ***A. archangelica***.

This key is reproduced in the Romanian literature (Beldie, 1972) for the Ciucaş Mountains plants (Ciuca, Beldie, 1989) and is built as follows:

1. petiole and rachis on the upper side of the leaf channelled; hairy peduncle on the entire length (erroneous "villose pubescent") – ***A. sylvestris*** subsp. ***montana***;
1. petiole cylindrical, only the rachis channelled, hairy pendulcle only on the apex – ***A. archangelica***.

According to some French botanists (Durin *et al*., 1989), the main differences between the two species are as follows:

1. flowers green, terminal leaflet trilobed, leaves hairy abaxial, plant very aromatic – ***A. archangelica***;
1. flowers whitish, terminal leaflet not lobed; leaves glabrous, plant non- or weak odorant – ***A. sylvestris***.

Unfortunately, Romanian botanists, including us, did not notice that the leaves of *A. archangelica* are hairy abaxial, but on the contrary we have noticed that those of *A. sylvestris* are adaxial setulose on the veins.

If we summarize the differences between *A. sylvestris* and *A. archangelica*, by various authors (Todor, 1958; Voroshilov *et al*., 1966; Rothmaler, 1966; Cannon, 1968; Beldie, 1972; Durine *et al*. 1989; Ciuca, Beldie, 1989; Yankova, Cherneva, 2007 etc.) and after our observations we obtain the following:

Table 1

Angelica sylvestris s.l. and *Angelica archangelica* subsp. *archangelica* –
a comparison of morphological characters

Angelica sylvestris s.l.	***Angelica archangelica*** subsp. ***archangelica***
1. Plant dark green, non- or weak odorant	1. Plant pale green, strongly odorant
2. Petiole deeply *channelled* and sometimes ± cylindrical (Fig. 2).	2. Petiole *cylindrical*, with a very narrow flat area
3. Leaves hairless abaxial	3. Leaves abaxial hairy (?)
4. Veins adaxial setulose	4. Veins hairless or poorly setulose on the edge
5. Terminal leaflet usually *not lobed*, sometimes subterminal leaflets long decurrent and connate	5. Terminal leaflet *trilobed*
6. Peduncle hairy on the entire length (Fig. 4)	6. Pendulcle hairy only on the apex
7. Umbels c*orymb shaped* (hemispheric or near spheric)	7. Umbel *spheric*
8. Flowers whitish (– pink)	8. Flowers greenish (– yellowish)
9. Mericarp *brownish*	9. Mericarps pale yellow or *whitish* (Fig. 2d)
10. Lateral wings ± the same width as mericarp (1.5 mm), rarely narrower, sometimes thickened at the base (Fig. 6b, 6c)	10. Lateral wings narrower than mericarp (0.75 mm)
11. Dorsal wings obtuse, prominent, mat, with the midrib in the middle (cross-section)	11. Dorsal ribs acute, filiform**,** shiny, slightly curved, distanced, with the midrib in the apex (cross-section)
12. Stylopod *prominent*	12. Stylopod *flat*
13. Secretory channels – 6, thick*,* visible externally as black stripes	13. *Secretory channels numerous* (approx. 20), *filiform*, *not visible externally*
14. Pericarp *attached* to the seed	14. Pericarp *not attached* to the seed
15. Seed width about 1.9 mm	15. Seed width about 2.75 mm
16. Stipels oftent present at lower leaves (Fig. 5)	16. Stipels absent

Table 2

Angelica sylvestris subsp. *sylvestris* and *Angelica sylvestris* subsp. *montana* –
the differences in vegetative organs

Subsp. ***sylvestris***	Subsp. ***montana***
1. All umbels hemispheric	1. Almost perfectly spherical central umbel at least
2. Petiole channelled (Fig. 3, Fig. 4)	2. Petiole cylindrical with an adaxial flat area
3. Vaginas moderately vesicular	3. Vaginas highly vesicular
4. Subterminal leaflets non- or narrowly decurrent	4. Subterminal leaflets clearly decurrent and connate
5. The base of the lateral fruit wing 0.25 to 0.3 mm thick (in cross-section) (Fig. 1a)	5. The base of the lateral fruit wing 0.5 mm thick (in cross-section) (Fig 1b)
6. Stem 1–2 cm thick	6. Stem up to 3–4 cm thick
7. Dorsal ribs distant	7. Dorsal ribs near
8. Grows at lower altitudes, at the base of mountains (*Filipendulo-Petasition*, *Alno-Ulmion*, *Salicion albae*)	8. Usually grows at higher altitudes (*Adenostylon*)

The difficulty arises in interpreting the material from higher altitudes, very robust, with the habit of *A. archangelica*, fooling those botanists that have not examined the mature fruits. The fruit is typical to *A. sylvestris*, brownish in colour, wide lateral wings, 6 secretory channels visible externally, high dorsal ribs, with subterminal rib and connate pericarp with the seed. Vegetative domain, robust in size, with thick stem up to 4–5 cm in diameter, cylindrical petiole, strongly vesicular vaginas and more or less spherical umbels, leads erroneously to *A. archangelica* (like in Flora Romaniae Exiccata, nr. 1302; Fig. 8), but the leaflet ribs are setulose adaxial, as in *A. sylvestris*. This material represents *A. sylvestris* subsp. montana, which can be separated, with some difficulty, from *A. archangelica*, but also from subsp. *sylvestris*.

The identification of Angelica taxa is hampered by the description of the hybrid *A.* × *mixta* Nyár. 1958, Fl. Pop. Rom. Rep. 6: 659 (Add.) (*A. archangelica* × × *A. sylvestris*) only by its vegetative organs characters: robust, swollen vaginas ± ± globular umbel (from *A. archangelica*), hairy peduncle, scabrous veins (from *A. sylvestris*). This plant can be classified exactly as *A. sylvestris* subsp. *montana*!

Fruits of *Angelica pancicii* Vand. from Rhodope (Bulgaria) were also analyzed based on the contradiction that some authors consider it synonymous to *A. sylvestris* (Cannon, 1968), others kept as separate species (Peev, 1982) because of the vesicular vaginas and the number of bracteoles (8–12). Fruits are identical to those of *A. sylvestris* subsp. *montana*, brownish, 5 to 3.75 mm, lateral wings from 1.25 to 1.35 mm thick and wide, obtuse and close dorsal ribs, with six secretory channels externally visible (Fig. 1b).

CONCLUSIONS

Taxa Angelica can be correctly separated only by mature fruit material. The lateral wings of the mericarp are spongy in *A. sylvestris* subsp. *montana*, not in *A. archangelica*, as stated in the key of the Flora Europaea (Cannon, 1968), and proven by *A. pancicii* mericarps (Fig. 2c). The following taxa occur in the Romanian flora:

1. **Angelica sylvestris** L. 1753

a. subsp. **sylvestris** (= var. *vulgaris* Avé-Lall. 1842, var. *typica* Beck 1892)

b. subsp. **montana** (Brot. 1804) Arcang. 1882 (*A. montana* Brot. 1804, *A. sylvestris* var. *elatior* Wahlenb. 1814, *A. alpina* Schur 1866 , *A. pancicii* Vand. 1891 (Fig. 1c), *A.* × *mixta* Nyár. 1958).

2. **Angelica archangelica** L. 1753 (= *Archangelica officinalis* Hoffm. 1814)

a. subsp. **archangelica** (= *Angelica sativa* Mill. 1768, *Archangelica sativa* Besser 1822)

3. **Angelica palustris** (Besser) Hoffm

Key to taxa identification

1. Stem costate-sulcate; terminal leaflet non-decurrent; petals unguicular; calyx teeth evident – ***A. palustris***

1. Stems smooth, terminal leaflet usually non-decurrent; petals non-unguiculate, calyx teeth absent

2. Fruits brown, with six large secretory channels, visible as black stripes, usually as wide as the lateral wings of the fruit, pericarp attached to the seed, flowers white to pink; ribs adaxial setulose, terminal leaflet usually undivided – ***A. sylvestris***

3. Petiole channelled; hemispherical umbels, subterminal leaflets non- or narrow decurrent, base of lateral wings from 0.25 to 0.30 mm thick – subsp. ***sylvestris***

3. Petiole cylindrical, with an adaxial narrow flat area, at least central umbel ± spherical; subterminal leaflets evident decurrent and connate, lateral wing base 0.5 mm thick – **subsp. *montana***

2. Fruit pale yellow or whitish (Fig. 2d), with numerous secretory channels not visible externally (Fig. 1d); lateral wings narrower than the fruit; pericarp not attached to the seed; flowers yellow-greenish; ribs adaxiale, ± glabrous, terminal leaflet usually tripartite – ***A. archangelica*** subsp. ***archangelica***.

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INDUCTION OF QUANTITATIVE VARIABILITY THROUGH EMS TREATMENT IN *VIGNA UNGUICULATA*

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The present study was conducted to evaluate the effect of EMS on different morphological yield parameters of cowpea in M_1 and M_2 generations. Both positive and negative shifts in mean values of yield parameters were recorded as a result of EMS treatment. Cowpea being a self-pollinated vegetable crop has very limited genetic variability therefore induced mutation can provide an additional source of mutation in a recent plant breeding programme. Hence, an experiment was conducted to evaluate the extent genetic variability in morphological quantitative characters in M_1 and M_2 generation following mutagenesis with EMS. By inducing mutation in cowpea, it may be possible to identify new beneficial traits for higher yield. Thus aim of the present study is to identify and select mutants with useful morphological attributes.

Key words: ethyl methane sulfonate; cowpea; quantitative traits.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an important leguminous crop, usually grown in developing countries of the world including India (Duke, 1990). It is utilized as grain, vegetables (leaves and immature pods) and fodder for livestock. It is a major cheap source of protein in human diets with the grains containing about 23–25% protein and 64% carbohydrates (Bressani, 1985). The proteins in cowpea seeds are rich in lysine and tryptophan as compared to cereal grains (Rachie, 1985).

Recently, induced mutagenesis has been widely employed to create desired genetic variability in crop improvement (Yaqoob and Rashid, 2001). The mutagens may cause genetic changes in an organism, break the linkage and produce many new promising traits for improvement of crop plants (Shah *et al*. 2008). Several chemical mutagens are frequently used for induced mutagenesis in crop respectively viz. ethyl methane sulphonate (EMS), ethylene amine (EI), methyl nitroso urea (MNU), N-nitroso-N-methyl urea (NMU), and ethyl nitroso urea (ENU) (Tah, 2006). Among the chemical mutagens EMS is reported to be the most effective and powerful mutagens (Minocha *et al.* 1962; Hajra *et al*. 1979). EMS has been found to be more effective and efficient than physical mutagens in crop like cow pea (Jhon, 1999), lentil (Gaikwad and Kothekar, 2004). In plants, EMS

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usually causes point mutation causing A.T = G.C base pair transition (Okagaki *et al.*, 1991). Chemomutagens include a broad variation of morphological and yield structure parameters in comparison to normal plants (Rao and Rao 1983).

MATERIAL AND METHODS

Seeds of *Vigna unguiculata* L. Walp var. K 5269 were obtained from Chandrasekhar Azad Agricultural University, Kanpur, India. The seeds were treated with 0.5% for EMS doses at different durations (1 h, 3 h and 5 h). After treatment of EMS, seeds were thoroughly washed to terminate the residual effect of mutagenic chemicals. The treated seeds as well as control seeds were sown in the experimental pots in replicates to raise the population. At maturity all the surviving M_1 fertile plants were harvested separately and sown in the next season in respective experimental pots to raise the M_2 population. The morphological data viz. plant height, leaf length and leaf breath of pot grown plants were taken after 30 days of sowing. However, morphological data of pod size and seeds per pod were taken after maturity of pods for studying both generations. The respective control and treatment progenies were screened several times for morphological mutation throughout the crop duration.

RESULTS

From the present investigation it is revealed that the different yield parameters, viz. plant height, leaf length, leaf breath, pod size, seeds/pod considerably increased in 1h EMS treated plants with respect to their control in both consecutive generations.

The plant height was found to be the highest in 1h EMS treated plant population than in the control in M_1 and M_2 generation (Figs. 1–2). In M_1 plant height ranged from 43.48 to 36.74 cm (Table 1) whereas in case of M_2 it ranged from 37.08 to 43.48 cm (Table 2). The result indicated that the EMS could cause both positive and negative variability in plant height.

The range of length of mature leaf was 8.82–10.68 cm in M_1 and 10.68–10.08cm in M_2 under different doses of EMS. The highest value of it was recorded in 1h EMS treated sets of M_2 generation. The range of leaf was 5.69–6.96 and 5.88–6.88 cm for M_1 and M_2 generation respectively. The 1 h EMS treated plants of M_1 and M_2 showed the highest value of leaf breath. However, the lowest value of it is observed at 5h EMS treated set in M_1 generation.

The size of pod showed a significant difference among all the parameters. The highest value of pod size was obtained in 1 h EMS treated plants of M_2. The reduction in mean of pod size found in 3 h and 5 h EMS treated sets of M_1 and M_2 may be attributed to induction of more mutation with negative effects.

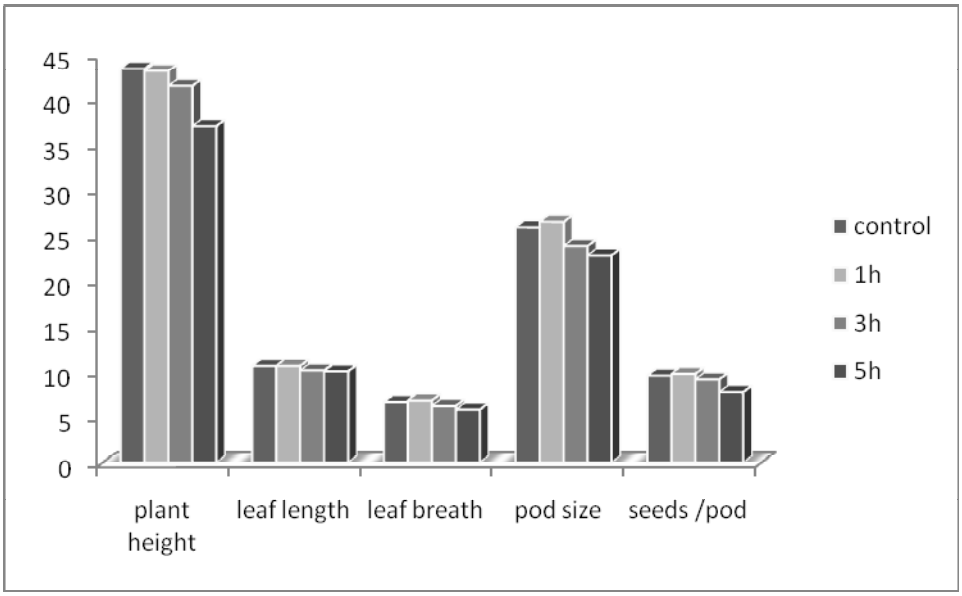

Fig. 1. Graphical representation of Yield parameters of EMS treated meiotic cells in M1 Generation.

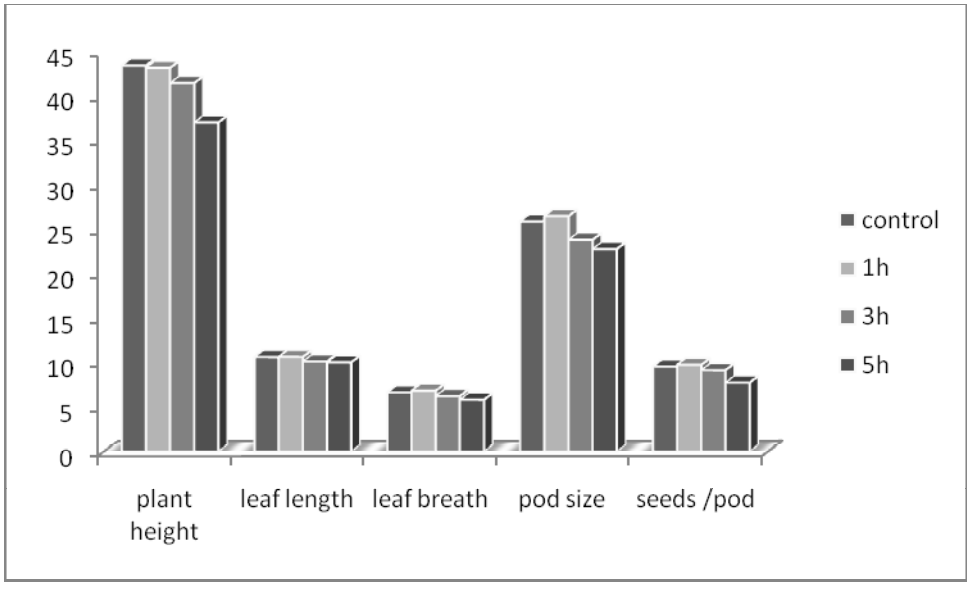

Fig. 2. Graphical representation of Yield parameters of EMS treated meiotic cells in M2 Generation.

Table 1

Different Yield parameters of EMS treated meiotic cells in M_1 Generation

Yield parameter	Mean	Variance	Coeff. of variance	Standard deviation	Standard error
Plant height					
Control	43.26	6.683	5.976	2.585	1.156
1h	43.48	5.217	5.241	2.284	1.021
3h	39.90	1.160	2.699	1.077	0.481
5h	36.74	3.253	4.909	1.804	0.807
Leaf length					
Control	10.583	0.189	4.115	0.436	0.178
1h	10.680	0.652	7.561	0.807	0.361
3h	9.640	0.268	5.370	0.517	0.232
5h	8.820	0.242	5.577	0.492	0.220
Leaf breath					
Control	6.800	0.195	6.934	0.442	0.197
1h	6.960	0.123	5.039	0.351	0.157
3h	6.260	0.088	4.739	0.297	0.133
5h	5.960	0.068	4.376	0.261	0.117
Pod size					
Control	25.54	4.458	8.267	2.111	0.944
1h	26.28	5.974	9.279	2.438	1.090
3h	23.56	2.453	6.647	1.566	0.700
5h	21.26	10.228	15.043	3.198	1.430
Seeds per					
pod	9.400	5.300	24.491	2.302	1.030
Control	9.800	4.700	22.121	2.167	0.970
1h	9.200	1.700	14.172	1.303	0.583
3h	7.400	1.300	15.407	1.140	0.509
5h					

Table 2

Different Yield parameters of EMS treated meiotic cells in M_2 Generation

Yield parameter	Mean	Variance	Coeff. of variance	Standard deviation	Standard error
Plant height					
Control	43.48	3.907	4.546	1.977	0.884
1h	43.22	4.412	4.850	2.100	0.939
3h	41.52	5.377	5.585	2.319	1.037
5h	37.08	3.257	4.867	1.804	0.807
Leaf length					
Control	10.68	0.097	2.917	0.311	0.139
1h	10.69	0.268	4.766	0.517	0.231
3h	10.18	1.012	9.881	1.005	0.499
5h	10.08	1.457	11.975	1.207	0.540

Table 2

(continued)

Leaf breath					
Control	6.68	1.012	15.059	1.005	0.450
1h	6.88	0.852	13.416	0.923	0.412
3h	6.28	0.432	10.466	0.657	0.294
5h	5.88	0.337	9.872	0.580	0.259
Pod size					
Control	25.94	4.328	8.010	2.080	0.930
1h	26.54	2.843	96.353	1.686	0.754
3h	23.88	9.572	12.956	3.093	1.383
5h	22.84	6.808	11.424	2.609	1.166
Seeds per pod					
Control	9.60	1.300	11.877	1.140	0.509
1h	9.80	2.200	15.135	1.483	0.663
3h	9.20	0.700	09.094	0.836	0.374
5h	7.80	3.700	24.667	1.924	0.860

The range of number of seeds/pod was 7.40–9.80 for M_1 and 7.80–9.60 for M_2 plants. The values of seeds per pod in 1 h EMS treated sets of M_1 and M_2 generations were statistically same.

DISCUSSION

Plant breeding along with advances in agronomic and production practices has played a major role in advancing grain yield per hectare over the past 50 years (Borlaug, 1983). Pulses generally have yield per hectare lower than cereals. Plants with increased yield parameters have a promising possibility of improving total yield per hectare.

In Tables 1, 2 it was revealed that a wider range of variation was observed for all morphological traits studied. It suggested the presence of sufficient variations for these morphological yield parameters to exploit the variability. Similar variation was recorded in barley for plant height, number of kernel per spike and spike length by mutagenesis of gamma rays (Qritz *et al.*, 2001). Morphological traits such as plant height, peduncle per plant, 1000 seed weight and seeds/pod were more increased than their respective control with effect of EMS and gamma rays recorded in M_1 generation of cowpea (Odeigah, 1998). According to Balyon and collaborators (1991) and Wicks and collaborators (2004), plant height has been shown to be an important trait for predicting the competitive ability of wheat cultivars. Wani and Anis (2004) reported improved yield parameters, namely, plant height, number of branches/plant, 100 seed weight and plant yield with effect of EMS and gamma rays in M_2 generation of chickpea. Arulbalachandran and

Mullainathan (2009) reported improvement in quantitative traits in M_2 generation in black gram induced by gamma rays.

The investigation showed that most of different yield parameters in 1 h EMS treated sets of M_1 and M_2 generation increased as compared to their respective controls due to effect of EMS on genome induce variability. It indicates that improvement in quantitative traits would be possible through EMS.

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INHIBITION OF STEROL BIOSYNTHESIS IN TOMATO PLANT RESULTING IN ANTIVERMIN PROTECTION

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Plant sterols are important ingredients of the plant's cell membrane or composition, regulate the fluidity and permeability and modulate the activity of bond enzymes required for growth and development. *In vitro*, using the methods of cell selection to study the effect of morpholines fungicide fenpropimorph on tomato plant (*Lycopersicum esculentum* var. pritchard), numbers of differentiated and un-differentiated tissues were affected. A few number of organogenic calluses and primary calluses were observed growing at the lethal concentration (50 mg L-1). Organogenic calluses were more sensitive to the effect of fenpropimorph than primary ones. Higher decrease in the dry weight values and sterol contents was observed in fenpropimorph growing calluses, Seed germinated and regenerant fenpropimorph (20 mg/L-1) treated plants showed a higher decrease in the sterol contents compared with control ones. Fenpropimorph regenerant plants showed a higher decrease in the sterol contents than its seed germinated ones. Fenpropimorph (40 mg/L-1) treated calluses were examined as exogenous supply of sterol in the diet of *D. melanogaster*; they decreased the number of progenies.

Key words: plant callus, fenpropimorph, fungicide, *Lycopersicum esculentum*, sterols.

INTRODUCTION

Higher plant cells synthesize a complex array of sterol mixture in which the Δ5-sterols (*i.e.* sitosterol, stigmasterol and campesterol) are often predominant (Hartmann, 1998; Schaller, 2003). Stigmasterol regulates the activity of the Na+/K+-ATPase in plant cells (Schaller, 2003; Fernandes and Cabral, 2007) and may be required specifically for cell differentiation and proliferation (Hartman, 2004; Volkman, 2005). Certain sterols, such as campesterol, in minute amounts are precursors of oxidized steroids (brasinosteroids) that act as growth hormones which have crucial importance for growth and development, (Hu, *et al.*, 2000; Noguchi *et al.*, 2000).

Fungicide fenpropimorph is a strong inhibitor of sterol biosynthesis pathway in higher plants and other eukaryotes (Maillot-Vernier *et al.*, 1991; He *et al.*, 2003). Morpholines and their analogues are assumed to inhibit an isomerisation step and a reduction step in sterol biosynthesis pathway of higher plants (Campagnac *et al.*, 2008). Fenpropimorph inhibits two different steps in sterol biosynthesis: the

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opening of cyclopropane ring of cycloeucalenol and the demethylation of C14-obtusifoliol (Campagnac *et al.*, 2009). The inhibitory effect of fenpropimorph on plant growth may be attributed to its effect on sterol biosynthesis pathway or its accumulation in plant tissues (Taton *et al.*, 1987).

Some insects are unable to de novo synthesize steroids responsible for moulting, ovipositor and wing pattern polyphenisms depending upon the exogenous supply obtained in their diet (Behmer and Nes, 2003; Song *et al.*, 2005; Palli *et al.*, 2005). Deficiency in exogenous sterols in the diet of some insects leads to inability to complete morphogenesis process (Hatle, 2003; Lutova *et al.*, 1994a). On quantitative basis, the bulk of sterol is required for insects for satisfactory growth and development (Lutova *et al.*, 1994b; Carlos *et al.*, 2005). Based on this the present investigation is an attempt to study the effect of fenpropimorph on the sterol biosynthesis in some forms of tomato plant tissue culture, as this effect was correlated to obtain a normal sterol content it does not meet insect requirements.

MATERIALS AND METHODS

Culture media. *In vitro*, for all experiments of induction, multiplication and regeneration of tomato callus cultures there was used MS (Murashige and Skoog, 1962) solid medium, supplemented with 3% sucrose and growth regulators, solidified with 1% Difco Bacta agar. The pH of the medium was adjusted with KOH or HCl to 5.8 before autoclaving.

Reagents. Fenpropimorph: cis-4-[3-[4-(1,1-dimethylethyl)phenyl-2-methyl-propyl]-2,dimethylmorpholine was kindly provided from the Agricultural Research Center in Cairo. Fenpropimorph was dissolved in a mixture of EtOH and DMSO (1:9) and was added to sterilized MS medium. The maximum concentration of the solvents was 0.01%.

Plant material preparation. Seeds of tomato (*Lycopersium esculentum* var. pritchard) were surface sterilized in ethanol 75% (v/v) for 2 min, then dipped in commercial bleach solution (1.05% hypochlorite solution) for 20 min, then they were rinsed three times in sterile distilled water and placed on MS (Murashige and Skoog, 1962) medium containing 3% sucrose without growth regulators, solidified with 1% Difco.Bacto agar. Germination was conducted in the dark at 25 ±2 °C for 1 week and another week in the light. For callus induction 2 weeks old seedlings excised leaves were inoculated on MS solid medium supplemented with 2mg L-1 IAA, 1 mg L-1 Kinetin and 3 % sucrose, incubated in the dark at 25 ±2O °C. Calluses were recorded for explants after 4 weeks from culture initiation. Recorded calluses were multiplied on the same solid medium (MS), which supplemented with the same growth regulators (2 mg L-1 IAA and 1 mg L-1 Kinetin) under the same standard culture conditions of temperature and darkness as mentioned before. For, organogenic callus obtaining, leaf derived calluses were cultured on MS solid

medium supplemented with I mg lAA L-1 and 2 mg L-1 kinetin, then they were maintained at the standard conditions of photoperiod regime (16 h/day) and an irradiance of 50 μE m-2 S-1 and temperature (25 ± 2 °C). After 4 weeks adventitious buds formation was observed. For the effect of fenpropimorph on the variability and growth of tomato calluses there were used micro-calluses (1.5––2.0 mm2) of both primary callus and organogenic callus. Primary micro-calluses were cultured on the multiplication medium (MS solid medium) supplemented with the growth regulators of callus formation (2 mg L-1 lAA and 1 mg L-1 kinetin) and different concentrations (0.0, 10, 20, 30, 40, 50, and 60 mg L-1) of the selective agent (fenpropimorph), then they were incubated in darkness of 25 ±2 °C, while organogenic micro-calluses were cultured on the regeneration medium (MS solid medium) supplemented with the growth regulators (1 mg L-1 lAA and 2 mg L-1 kinetin) of organogenic callus obtaining and the same different concentrations of the selective agent (fenpropimorph), then they were incubated in the light regime of 16 h/d and light intensity of (40 μE m-2 s-1) for 4 weeks at the standard conditions of temperature as mentioned before. Five plates were used for each treatment, each contained with 20 micro-calli. The experiment was conducted three times with the same number of replicates), in which the second subculture lacked the selective agent (fenpropimorph). Fenpropimorph treated calluses (40 mg/L-1) were harvested after 4 weeks and analyzed to determine their sterol content.

INHIBITION OF STEROL BIOSYNTHESIS OF SEED GERMINATED AND REGENERANT PLANTS

Sterilized seeds of tomato (*Lycopersicum esculentum* var. pritchard) were soaked in fungicide solution (20 mgL-1), then they were transferred into 250 ml glass jars contained with MS solid medium lacked growth regulators, supplemented with 0.0 and 20 mg L-1 fungicide fenpropimorph. Germination occurred in controlled conditions as mentioned before without humidity control. The same experiment was conducted also to the regenerant plants, where organogenic callus excited shoots were transferred into the same solid medium (MS) which lacked the growth regulators and supplemented with the same concentration (0.0 and 20 mg L-1) of fenpropimorph at the same standard conditions of light regime and temperature as mentioned before.

STEROL ANALYSIS

Total lipids were fractionated according to the method of Farag *et al.*, (1986). Fatty acids and unsaponifiables were separated, fatty acids were extracted three times with peroxide free diethyl ether. Then unsaponifiables were analyzed by GLC analysis according to A.O.A.C. (1986).

BIOLOGICAL ASSAY

The biological assay for *Drosophila* was conducted as described by Lutova *et al.*, (1994a). In which it was used, *Drosophila melanogaster*, Kantom C., and yeast mutant lacked Δ7–Δ5 isomerase were kindly provided from Genetics and Breeding Department, St. Petersburg State University, Russia.

STATISTICAL ANALYSIS

Data were analyzed according to SAS (1993) using the L1 linear model.

RESULTS

We defined the lethal concentration of cultures, as the lowest concentration of inhibitor which prevented the growth of micro-calluses treated during 4 weeks. Absence of further growth was indicator of lethality (Table 1). Organogenic micro-calluses were more sensitive to the inhibitor effect of the fungicide compared to the other ones. Most of primary microcalli and organogenic microcalli failed to grow at the sub-lethal concentration (40 mg L-1). High significant differences were observed in the growth capacity of calluses at the lethal concentrations compared with control ones, also among the weight values of the growing micro-calluses in the different concentrations of the selective agent (fungicide fenpropimorph), since the inhibitory effect of fungicide as well as the concentration increased. A few numbers of organogenic micro-calluses and primary micro-calluses were observed growing at the lethal concentration (50 mg L-1), but these micro-calluses seemed brown and weak for their phenotype. They died during the subculture into a fenpropimorph free medium. GLC analysis for the un-saponifiable matters of the developed calluses of tomato under the influence of the sub-lethal concentration of fenpropimorph (40 mg L-1) revealed a higher decrease in the final sterol (sitosterol, stigmasterol and campesterol) contents compared to control ones (Table 2) and a dramatic quantitative reduction in the total sterols content of the usual sterols of fenpropimorph-treated calluses. The decrease in usual sterol contents of fenpropimorph treated undifferentiated (primary calluses) and differentiated (organogenic calluses) tissues was more pronounced in organogenic calluses than in primary calluses (Table 2). Similar result was obtained in the case of triadimefon (20 mg/l)-developed plants (Table 3), where, the major plant sterols (sitosterol, stigmasterol, campesterol) contents of tomato seedlings and regenerative plants showed a higher decrease in their contents compared to control ones. There was a dramatic quantitative reduction in the percentages of the total sterols content compared with control ones. The effect of fenpropimorph was more pronounced in

shoots than in roots. The decrease in usual sterol contents of fenpropimorph treated plants was more pronounced in the regenerant plants than in intact ones. The results of using the homogenate of the developed calluses under the influence of fenpropimorph (40 mg L-1) for three consecutive subcultures, but the second subculture lacked the selective agent (fenpropimorph) as sole source of exogenous sterol in the diet of *D. melanogaster* mutant (Fig. 1) showed that the number of insects produced in the first and second generation decreased compared to control ones.

Table 1

Effect of fenpropimorph concentrations on the viability and growth of tomato callus cultures.
Data represent means ±SD of 100 replicates / treatment in three reported experiments.
Followed by LSD test at alpha <0.05

Treatment	**Viability and growth of Calluses**			
	Primary calluses		**Organogenic calluses**	
	% of growing calluses	**Callus dry weight (mg/callus)**	**% of growing calluses**	**Callus dry weight (mg/callus)**
Control	99.33 ± 0.58	61.94 ± 3.94	98.33 ± 1.53	51.52 ± 1.52
10	82.67 ± 3.06	52.62 ± 2.51	76.00 ± 3.00	44.35 ± 3.20
20	58.00 ± 1.73	32.45 ± 2.73	52.67 ± 2.08	22.81 ± 2.58
30	36.00 ± 2.00	17.28 ± 1.70	22.33 ± 2.52	12.81 ± 0.89
40	08.67 ± 2.00	05.99 ± 0.97	05.67 ± 2.51	04.58 ± 0.46
50	02.67 ± 0.58	01.62 ± 0.54	01.33 ± 0.58	01.36 ± 0.32
60	0.0	0.0	0.0	0.0
LSD	2.35	3.08	2.75	2.42

Table 2

Sterol contents of fungicide fenpropimorph (40 mg\L) treated calluses.
Percentage was calculated relative to the total
percentage of unsaponifiable matters

Sterol	Relative % of Δ^5 – Sterol Contents			
	Callus		Organogenic callus	
	Control	Treated	Control	Treated
Sitosterol	5.2	3.4	5.8	2.9
Stigmasterol	5.3	3.2	5.9	2.5
Campesterol	3.5	1.4	3.2	1.4
Chlosterol	4.6	3.3	4.9	1.8
Total	17.6	11.3	19.8	8.6

Table 3

Sterol content of germinated seedlings and regenerated plants on MS medium supplemented with 20 mg/L fenpropimorph. Percentages were calculated relative to the total percentage of unsaponifiable matters

Sterols	Relative % of Δ5 – Sterol Contents							
	Seedling				Regenerant Plant			
	Roots		Shoots		Roots		Shoots	
	control	treated	control	treated	control	treated	control	treated
Sitosterol	4.4	3.7	6.3	1.2	5.2	2.4	5.7	2.2
Stigmasterol	4.2	2.5	4.2	3.1	3.7	2.1	4.3	1.7
Campesterol	3.3	1.3	3.5	2.4	2.8	1.7	3.6	1.3
Chlosterol	4.6	2.7	4.9	2.6	4.7	2.4	4.2	2.7
Total	16.5	10.2	18.9	9.3	16.4	8.6	17.8	7.9

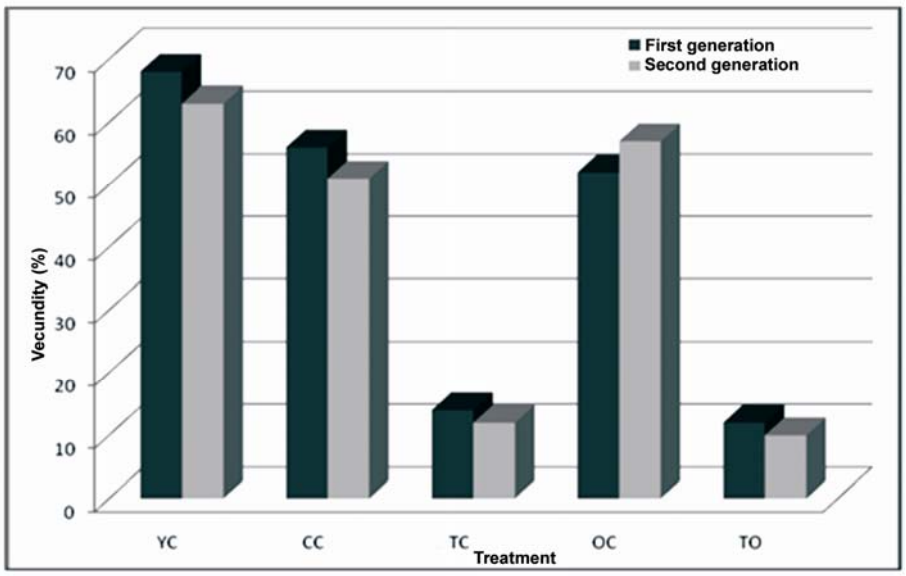

Fig. 1. Plant stocks as a source of exogenous sterol of *D. melanogaster*, Y.C = Yeast Control, C.C = Callus Control, T.C = Treated Callus, O.C = Organogenic Callus, T.O = Treated Organogenic Callus. Data represent means of three reported experiments.

DISCUSSION

Morpholine fungicides, known to be a strong inhibitor of sterol biosynthesis in higher plant cells (Campagnac *et al.*, 2008), is used as screening agent in agriculture. The decrease of dry weight values among the fenpropimorph treated

calluses as well as the concentration increase may be attributed to the inhibitory effect of the fungicide fenpropimorph on sterol biosynthesis pathway, since plant sterols play an important role in the cell division, and its development. This observation is in agreement with those reported by Hartmann (1998); Carland and collaborators, (2002); Campagnac and collaborators (2009) supporting that, sterol plays a crucial role in the growth and development of plant cells. Stigmasterol may be required specifically for cell differentiation and proliferation (Carland, *et al.*, 2002; Hartman, 2004; Volkman, 2005). Campesterols, in minute amounts, act as growth hormones, which have crucial importance for growth and development (He, *et al.*, 2003; Hu, *et al.*, 2000; Noguchi *et al.*, 2000). Morpholine and its analogues are assumed to inhibit an isomerization step and a reduction step in sterol biosynthesis pathway of higher plant cells (Maillot-Vernier *et al.*, 1991; Schaller, 2003). Fenpropimorph inhibit two different steps in sterol biosynthesis: the opening of cyclopropane ring of cycloeucalenol and the demethylation of C14 obtusifoliol (Campagnac *et al.*, 2008). The inhibitory effect of fenpropimorph on tomato calluses growth may be attributed to the replacing of the typical sterols with intermediates (9ß-19 cyclopropyl sterols) during sterol biosynthesis pathway (Grandmougin *et al.*, 1989; He, *et al.*, 2003). This may be attributed to the inhibition of some steps in sterol biosynthesis pathway as mentioned before, or to the great accumulation of this inhibitor in these tissues or both together (Maillot-Vernier, 1991; Grandmougin *et al.*, 1989) who reported that systemic fungicide fenpropimorph accumulate 9ß-19 cyclopropyl sterols in place of Δ5-sterols (*i.e.* sitosterol, stigmasterol, and campesterol) which are normally produced in these plants. The biosynthesis pathway of brassinosteroids via two pathways from campesterol (Noguchi *et al.*, 2000; Carland *et al.*, 2002; Hu *et al.*, 2000), so the inhibition of campesterol biosynthesis may be affected by brassinosteroids biosynthesis. In plants, the diverse function of sterol derived brassinosteroids (BRs) as plant growth hormones was investigated by Altmann (1998); Jang and collaborators (2000), who reported that brassinosteroids play an important role in elongation, expansion and promoting xylem's differentiation in plant cells. The effect of fenpropimorph in this study was more pronounced in organogenic callus than in primary callus and in shoots than in roots. This might be due to the systemic action of this compound which caused a greater accumulation of the fungicide in these tissues (Hartmann, 2004). It was observed a higher decrease in the number of progeny of *D. melanogaster*. These results are in agreement with those obtained by Behmer and Nes (2003), Lutova and collaborators (1994ab) and Riddiford and collaborators (2000), who reported that some insects are unable to synthesize de novo the steroids nucleus depending upon the exogenous source whereas these insects use the phytosterols (Δ5sterols) as precursor of ecdysteroids which play an important role in the growth and development of insects. The decrease in the numbers of progenies might be attributed to the inhibitory effect of the fungicide, whereas fenpropimorph leads to complete replacement of the usual

sterols by unusual ones, this observation is agreed with those reported by Hatle (2003), Carlos and collaborators (2005) and Song and collaborators (2005), who reported that the selective inhibition of specific enzyme in the pathway of phytosterols biosynthesis can be used as a strategy to control insects development, where, some insects are unable to de novo synthesize sterol, depending upon the exogenous supply, which was obtained in their diet. On a quantitative basis, the bulk of sterol is required for insects for satisfactory growth and development (Carlos *et al.*, 2005; Palli *et al.*, 2005).

CONCLUSIONS

The present study proved that the fungicide fenpropimorph is a strong inhibitor of sterol biosynthesis pathway in tomato plant tissue culture and we can use the cell selection through the tissue culture technique to obtain some forms of plants with changeable sterol contents which affect the growth and development of pests.

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LOW DOSE OF GAMMA IRRADIATION ENHANCED DROUGHT TOLERANCE IN SOYBEAN

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Drought stress is the main limiting factor of soybean production. However, no work has been done on how application of low-dose of gamma rays could help to overcome water deficits during critical stages of soybean development. Gamma rays at doses 0.0 and 20 Gray (Gy), from a cobalt source (^{60}Co) with strength of 500 Ci and the dose rate of 0.54 Gy/min^{-1}, were applied to dry seeds of soybean before planting. Two levels of soil moisture (80% field capacity for well-watered control and 35% for drought-stressed treatment) were applied at pod initiation. Thereafter, the interaction effects of low dose of gamma irradiation and water stress on some growth, biochemical, anatomical and antioxidative parameters of soybean plants were investigated. Low dose of gamma irradiation increased biomass accumulation and seed yield for both treatments. Drought stress depressed chlorophyll content and photosynthetic activity ($^{14}CO_2$-fixation), while chlorophyll content, leaf water potential and photosynthetic activity of plants irradiated with gamma rays at a dose 20 Gy were greater than that of drought-stressed plants. Water deficit decreased the enzyme activities of phosphoenol pyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase. However, application of low dose of gamma irradiation (20 Gy) increased the activities of these enzymes, except for phosphoenol pyruvate carboxylase under drought stress. Gamma irradiation dose at 20 Gy increased the concentration of soluble sugars, protein and proline content and the activities of peroxidase and superoxide dismutase of soybean leaves when drought-stressed. However, it decreased the malondialdehyde concentration and electrical conductivity of leaves under drought stress. The following physicochemical characteristics of chloroplasts were chosen as indicators of drought-stressed effects: average size, and ultrastructure. The results suggest that gamma irradiation at dose 20 Gy can partly counterbalance the destructive effects of water deficits. This protective action led to an increase of chloroplast size reduced by drought treatment and rebuilt, to some extent, the chloroplast ultrastructure. Overall, the results indicated that pre-treatment with low dose of gamma rays (20 Gy) to dry seeds of soybean before planting can be used to enhance drought tolerance and minimize the yield loss caused by water deficits. Thus, it may be a useful management tool in afforestation projects in arid and semiarid areas as a promising technique for agricultural improvement.

Key words: drought stress, gamma irradiation, antioxidative enzymes, soybean, proline.

Abbreviations: MDA – malondialdehyde; POD – peroxidase; SOD – superoxide dismutases; ROS – reactive oxygen species; H_2O_2 – hydrogen peroxide; Ψ_{leaf} – leaf water potential; RuBPcase – ribulose-1,5-bisphosphate carboxylase/oxygenase; PEPcase – phosphoenol pyruvate carboxylase; Gy – Gray.

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INTRODUCTION

Soybean is one of the most economical and nutritious foods, which may be of help to counter malnutrition and under nutrition in developing countries. Drought limits plant growth on a large proportion of the world's agricultural land. Soybean is considered sensitive to drought stress, especially during critical periods of plant development (Liu *et al.* 2004). Water stress results in yield reduction by decreasing seed number and seed weight. Intermittent drought is most certain to occur during soybean ontogeny (Dornbos *et al.* 1989). Drought stress is the primary constraint for increasing soybean yield, particularly when it triggers an early switch from vegetative to reproductive development (Desclaux and Roumet 1996). Drought is an important environmental factor, which induces significant alterations in plant physiology and biochemistry. The most common symptom of water stress injury is the inhibition of growth, which is reflected in a reduction in the dry matter yield (Le Thiec and Manninen, 2003). Water deficit inhibits photosynthesis as it causes chlorophyll content alterations, harms the photosynthetic apparatus (Costa *et al.*, 1997). In addition, it modifies the activity of some enzymes and the accumulation of sugars and proteins in the plant (Gong *et al.*, 2005), resulting in lower plant growth and yield (Costa *et al.*, 1997). Drought stress was found to decrease the relative water content of plant leaves (Sánchez-Blanco *et al.*, 2002) and total chlorophyll (Shaddad and El-Tayeb, 1990), increase the accumulation of H_2O_2, lipid peroxidation, soluble proteins and free amino acids, including proline, in various plants (Gunes *et al.*, 2008). Drought induces the generation of reactive oxygen species (ROS), causing lipid peroxidation, and consequently membrane injury, protein degradation, enzyme inactivation and the disruption of DNA strands (Becana *et al.*, 1998). The MDA content is often used as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Smirnoff, 1993). Drought stress may lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species that are responsible for various damages to macromolecules and induce oxidative stress (Reddy *et al.*, 2004). The reduced activity of RuBPC induced by biotic and abiotic stresses is well documented in plants (Allen and Ort, 2001).

Gamma rays have been proved economical and effective as compared to other ionizing radiations because of their easy availability and the power of penetration. This penetration power of gamma rays helps in their wider application for the improvement of various plant species (Moussa, 2006). Sjodin (1962) reported that the material and energy necessary for initial growth are already available in the seed, and so the young embryo has no need to form new substances, but only to activate those already stored in the cotyledons. Low doses of γ-radiation may increase the enzymatic activation and awakening of the young embryo, which results in stimulating the rate of cell division and affects not only

germination, but also vegetative growth and flowering. Exposing the dry seeds to low γ-irradiation doses resulted in the increasing yield of some plants such as sunflower (Abo-Hegazi *et al.*, 1988) and *Ammi visnage* (El-Shafie, 1993). Also, Patskevich (1961) came to the conclusion that irradiation of seeds prior to sowing held a great promise from the viewpoint of its practical application in agriculture. It was generally agreed that low doses of gamma rays stimulate cell division, growth, and development of various organisms, including animals and plants. This phenomenon, named hormesis, was analyzed and discussed by various authors for various species (Korystov and Narimanov, 1997). Very low doses of gamma irradiation have been shown to stimulate plant growth (Watanabe *et al.*, 2000). Previous studies have shown that relatively low-doses ionizing irradiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Chakravarty and Sen, 2001). The objective of this work is to investigate whether pre-treatment with low dose of gamma rays (20 Gy) to dry seeds of soybean plants before planting may be a protectant agent to nullify the influence of drought stress.

MATERIAL AND METHODS

Plant material, growth conditions, and stress treatments. A homogeneous lot of soybean seeds (*Glycine max* L.), cv. Giza 83; was obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt. The caryopsis was kept at 4 °C. They were surface sterilized in 0.1 % (w/v) sodium dodecyl sulphate solution and then thoroughly rinsed with sterile deionized water. Dry seeds were exposed to doses of gamma irradiation, 0.0 and 20 Gy, using a gamma source (^{60}Co), Vinderen-Oslo 3-Norway, at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Cairo, Egypt) with strength of 500 Ci and the dose rate of 0.54 Gy/min. Seeds were allowed to germinate in pots 35 cm by 30 cm diameter. Each pot was filled with 15 kg sandy loam soil with 2.5% organic matter and available N, P and K concentration of 170, 80 and 200 mg kg^{-1}, respectively. Pots were arranged in a completely randomized design with two factors, two gamma irradiation doses (0.0 and 20 Gy) and two soil water levels (well-watered and drought-stressed) with 20 pots per treatment that were replicated four times. The 320 pots for the experiment were placed in a field sheltered from rain by a removable polyethylene shelter, at a day/night temperature of 24/18 °C, with 70% relative humidity, 14-h light and a photon flux density of 400 μmol $m^{-2}s^{-1}$. Cultural practices, such as weed control and irrigation, were performed as needed. Ten seeds were sown per pot. After the seedlings reached the first true leaf stage, they were thinned to four plants per pot. Two levels of soil moisture were applied by controlled watering beginning at pod initiation until harvest at full maturity. The well-watered and drought-stressed treatments were maintained at 80% and 35%

soil field capacity respectively, following the methods of Desclaux and Roumet (1996). The water deficit was initiated by withholding water. The pots were weighed daily to maintain the desired soil water levels by adding appropriate volumes of water. All biochemical estimations were carried out using three leaflets per newly expanded trifoliolate leaves. Samples were collected 10 days after the water treatment was applied, between 9:30 and 10:30 a.m., and kept in liquid nitrogen until analyzed. Effects of treatments on growth and yield were determined by measurement of accumulated biomass of the various organs. At harvest, the plants were removed carefully from the pots. The biomass and seed weights were determined with harvested organs being dried for 48 h at 70 °C.

Enzymes assay. Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBPcase, EC 4.1.1.39) was determined by Warren *et al.* (2000). Peroxidase (POD, EC 1.11.1.7), was assayed as given by Macheix and Quessada (1984). Superoxide dismutase (SOD, EC 1.15.1.1) was determined as described by Dhindsa *et al.* (1981). The activity of phosphoenol pyruvate carboxylase (PEPcase, EC 4.1.1.31) was determined as described by Gonzalez *et al.* (1998).

Chemical analysis. Total soluble protein contents were measured using Bradford's method (Bradford, 1976). Free proline was determined according to the method described by Bates *et al.* (1973). Lipid peroxidation was measured in terms of malondialdehyde content using the thiobarbituric acid reaction as described by Madhava Rao and Sresty (2000). Soluble sugars were evaluated using the anthrone method described by Fales (1951). Electrical conductivity was measured with a digital conductivity meter (JENWAY, Model 4070, Essex, England). Leaf water potential (Ψ_{leaf}) was measured with a pressure chamber (Model 3000, Soil Moisture Equipment Corp, Santa Barbara, CA, USA).

Total chlorophyll. The total chlorophyll content of fresh leaves was estimated following the method suggested by Barnes *et al.* (1992).

Photosynthetic activity ($^{14}CO_2$-fixation). Photosynthetic activity was measured in the atomic energy authority, Radioisotope Department, Cairo, Egypt, with the method of Moussa (2008). The seedlings from each treatment were placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive $^{14}CO_2$ was generated inside the chamber by a reaction between 10% HCl and 50 μCi (1.87×10^6 Bq) $NaH^{14}CO_3$ + 100 mg Na_2CO_3 as a carrier. Then the samples were illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by 80% hot ethanol. The ^{14}C was assayed from the ethanolic extracts in soluble compounds using a Bray Cocktail (Bray, 1960) and a Liquid Scintillation Counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises, Edinburgh, UK).

Isolation of chloroplasts. Chloroplasts were isolated from fresh leaves in chloroplast isolation buffer containing 50 mM Tris–HCl, 5 mM EDTA, 0.33 M sorbitol, pH 7.5 using the method of Block *et al.* (1983). Crude chloroplasts were

purified by centrifugation using 40%/80% Percoll gradient (Schwertner and Biale, 1973). Intact chloroplasts were collected from the gradients, diluted three to four times, and centrifuged at 2070 g for 2 min. Next, chloroplasts were resuspended in the isolation buffer and kept in darkness until future use. All procedures were carried out at 0–4 °C.

Electron transmission microscopy. For microscope observations, the lower epidermis was stripped off from the leaves. Samples were prepared as described by Coulomb *et al.* (1996). Briefly, after fixation in glutaraldehyde and post-fixation in osmium tetroxide, they were dehydrated in acetone and embedded in araldite. The sections, stained in uranyl acetate and lead citrate, were examined by transmission electron microscopy (TEM, Jeol Jem 1200 EX II, Tokyo, Japan).

Chloroplasts size determination. Chloroplast size distribution was determined by dynamic light scattering (DLS) technique (Beckmann, Coulter N4 Plus apparatus).The scattering angle was equal to 90°. A unimodal distribution was assumed for the mean particle size calculation.

Statistical analysis. All data were subject to ANOVA and means were compared using Duncan's multiple range tests ($P < 0.05$).

RESULTS

Average sizes of chloroplasts isolated from control soybean seedlings were about 1,200 nm and were not noticeably different from the size of the chloroplasts obtained from plants pretreated by 20 Gy dose of gamma irradiation (Fig. 1). Chloroplast sizes obtained from drought stressed plants were almost twice as small. This drastic decrease of the average chloroplast size was partly reduced in plants irradiated with gamma rays (20 Gy).

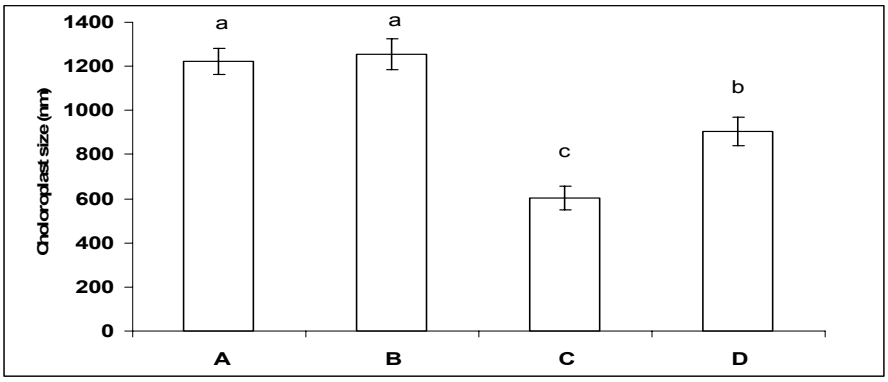

Fig. 1. DLS size of chloroplasts isolated from soybean leaves: (A) control; (B) plants irradiated with gamma rays (20 Gy); (C) drought stressed plants and (D) drought stressed plants pre-exposure to gamma irradiation (20 Gy). Values represent the means of four replicates ± SE. Different letters indicate significant differences ($P < 0.05$) between treatments.

Transmission electron microscopy of the chloroplasts of untreated soybean seedlings revealed the typical ultrastructure with well-organized envelope and internal membrane structure with normally developed grana and stroma thylakoids (Fig. 2A). The same chloroplast organization was observed in plants pretreated with gamma irradiation at dose 20 Gy (Fig. 2B). Chloroplasts of drought stressed plants showed an altered shape, with wavy grana and stroma thylakoids and enlarged intrathylakoidal spaces. In addition, envelope membranes were not visible at microscopic pictures of most chloroplasts (Fig. 2C). The changes in chloroplasts originated from drought stressed plants pre-exposure to low dose of gamma rays (20 Gy) were not as drastic as those observed for drought stressed plants only. However, some reorganization of the thylakoids and stroma was observed (Fig. 2D). Water deficits decreased the chlorophyll content by 12% and photosynthetic activity by 42%. However, the chlorophyll content and photosynthetic activity of plants irradiated with gamma rays (20 Gy) were higher than those of plants under drought-stressed conditions. Under well-watered conditions, the photosynthetic efficiency of the plants irradiated with gamma rays (20 Gy) was higher than of the control plants (Table 2). Water deficits decreased RuBPcase activity by 37% and PEPcase activity by 38%. However, plants irradiated with gamma rays (20 Gy) increased the activity of RuBPcase and PEPcase, except for PEPcase under drought stress (Table 1). Water deficit decreased the total soluble protein concentration by 9% (Table 2). However, the total soluble protein contents of plants pre-exposure to low dose of gamma rays (20 Gy) were higher than those of plants under drought-stressed conditions by 11%. Water deficits decreased Ψ_{leaf} (Table 2). The Ψ_{leaf} for well-watered plants was 0.45 to –0.50 MPa, while that for drought-stressed plants reached –1.8 to –2.3 MPa. Application of low dose of gamma rays (20 Gy) increased Ψ_{leaf} under drought-stressed conditions, but there is no difference in the Ψ_{leaf} between irradiated plants and control under the well-watered conditions. Water deficit treatment increased the concentrations of soluble sugar, proline, protein, the enzyme activities of POD and SOD and electrical conductivity of leaves (Tables 1, 2). Under drought-stressed conditions, pre-exposure to gamma rays increased the concentrations of soluble sugars, protein, proline and the enzyme activities of POD and SOD, but not the electrical conductivity of leaves or the concentration of MDA. For example, application of low dose of gamma rays (20 Gy) increased soluble sugar by 17% and proline by 12%, and increased SOD activity by 28% and POD activity by 30%, but MDA concentration decreased by 13% along with the electrical conductivity by 9% compared with the drought control (Tables 1, 2).

Table 1

Effect of gamma irradiation (20 Gy) on enzyme activities of RuBPcase (μmol CO_2 mg^{-1} protein min^{-1}), PEPcase (μmol CO_2 mg^{-1} protein min^{-1}), POD (units mg^{-1} protein) and SOD (units mg^{-1} protein), MDA (nmol g DW^{-1}), proline (μmol g DW^{-1}), and total soluble protein (mg g FW^{-1}) of soybean under well-watered and drought-stressed treatments[A]

Treatments	RuBPcase	PEPcase	POD	SOD	MDA	Proline	Protein
Well-watered	28.6[b]	2.9[b]	7.7[c]	2.8[c]	98[c]	33[c]	65[b]
Well-watered + γ-irradiation	29.7[a]	3.2[a]	7.8[c]	3.9[b]	102[c]	34[c]	71[a]
Drought-stressed	20.9[d]	2.1[c]	11.0[b]	4.2[b]	130[a]	45[b]	56[d]
Drought-stressed + γ-irradiation	23.1[c]	2.2[c]	14.3[a]	5.4[a]	115[b]	51[a]	62[c]

[A] Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P < 0.05$). Data are the means of four replicates.

Table 2

Effect of gamma irradiation (20 Gy) on photosynthetic activity (*KBq mg FW^{-1}), chlorophyll content (mg g FW^{-1}), the concentration of soluble sugar (mg g FW^{-1}), electrical conductivity (%), and Ψ_{leaf} (MPa) of soybean under well-watered and drought-stressed treatments[A]

Treatments	Photosynthetic activity	Chlorophyll content	Soluble sugar	Electrical conductivity	Ψ_{leaf}
Well-watered	16.8[d]	52.7[a]	117[d]	9.6[c]	-0.50[a]
Well-watered + γ-irradiation	19.7[c]	53.0[a]	149[c]	7.2[d]	-0.45[a]
Drought-stressed	11.8[b]	47.2[c]	182[b]	14.4[a]	-2.3[c]
Drought-stressed + γ-irradiation	14.9[a]	49.6[b]	213[a]	13.2[b]	-1.8[b]

[A] Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P < 0.05$).*kilo Becquerel (10^3 Bq). Data are the means of four replicates.

Water deficits decreased the dry weight of stems and leaves, total biomass and seed yield, but did not affect the dry weight of roots (Table 3). Application of low dose of gamma rays (20 Gy) increased the dry mass of roots, stems and leaves, and seed yield under both water levels, with the exception of the dry weight of

stems and leaves under drought stresses conditions. Under well-watered conditions, Gamma rays treatment also increased the dry weight of roots by 55%, stem plus leaves by 15%, total biomass by 21% and seed yield by 22% compared to unstressed control plants. Under drought-stressed conditions, gamma rays treatment also increased the dry weight of roots by 22%, stem plus leaves by 16%, total biomass by 19% and seed yield by 21% compared to the stressed control plants (Table 3).

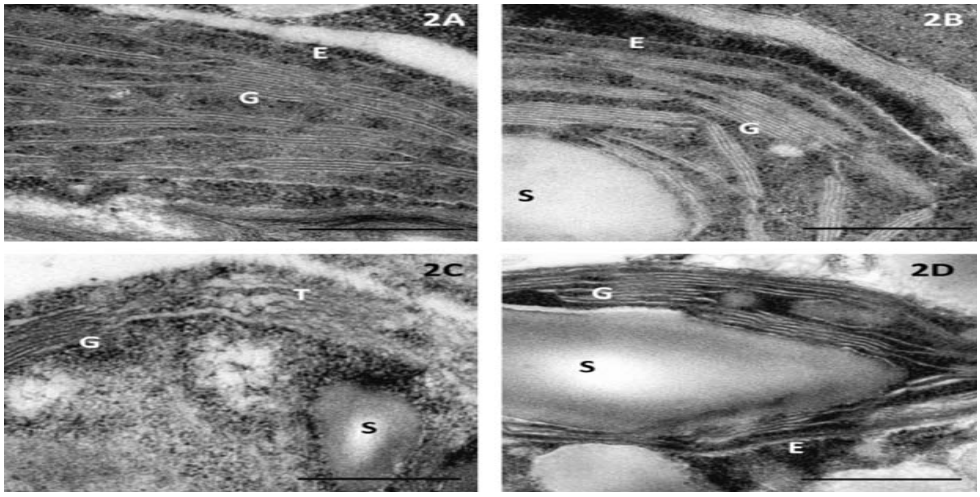

Fig. 2. Chloroplast structure of soybean leaves: (A) control; (B) plants irradiated with gamma rays (20 Gy); (C) drought stressed plants and (D) drought stressed plants pre-exposure to low dose of gamma rays (20 Gy). E – envelope, G – grana, S – starch and T– thylakoid.
Bars correspond to 200 nm.

Table 3

Effect of gamma irradiation (20 Gy) on the dry weight of roots, stems plus leaves, seed yield and total biomass of soybean (g/plant) under well-watered and drought-stressed conditions[A]

Treatments	Roots	Stems plus leaves	Seed yield	Total biomass
Well-watered	1.8[c]	11.6[b]	11.3[b]	24.7[b]
Well-watered + γ-irradiation	2.8[a]	13.3[a]	13.8[a]	29.9[a]
Drought-stressed	1.8[c]	8.1[c]	7.9[d]	17.8[d]
Drought-stressed + γ-irradiation	2.2[b]	9.4[c]	9.6[c]	21.2[c]

[A] Well-watered treatment was 80% of soil field capacity, and drought-stressed treatment was 35% of soil field capacity. Values followed by the same letter within columns are not significantly different according to Duncan's multiple range tests ($P < 0.05$). Data are the means of four replicates.

DISCUSSION

The effect of drought stress on the photosynthesis process is a subject of intensive investigation. The typical consequence of water deficits action on soybean seedlings consists of a decrease in chloroplast size and changes in the chloroplasts' inner structure, registered by microscopic observations. Drought stress causes a degradation of internal chloroplast membranes, leaving the integrity of chloroplast envelopes. Similar findings have bean reported by Dimitrina *et al.* (2002). The changes in chloroplasts originated from drought stressed plants pretreated with gamma irradiation (20 Gy) were not as drastic as those observed for drought stressed plants only. However, some reorganization of the thylakoids and stroma was observed. These results support the findings of Wi *et al.* (2007). Although no conclusive explanations for the stimulatory effects of low-dose gamma radiation are available until now, papers support a hypothesis that the low dose irradiation will induce the growth stimulation by changing the hormonal signaling network in plant cells or by increasing the antioxidative capacity of the cells to easily overcome daily stress factors such as fluctuations of light intensity and temperature in the growth condition (Kim *et al.*, 2004). In this study, the drought-stressed soybean plants irradiated with gamma rays (20 Gy) had higher biomass and seed yield than the stressed control plants. These beneficial effects resulted in higher leaf area, biomass production, grain yield and yield-related parameters in the treated plants (Moussa, 2006). Plants irradiated with gamma rays (20 Gy) before the onset of water stress in the present study improved leaf photosynthesis and chlorophyll content of soybean during the period of water stress. Abu *et al.* (2005) stated that an increase in chlorophyll a, b and total chlorophyll levels was observed in *Paulownia tomentosa* plants that were exposed to gamma irradiation. The plants irradiated with gamma rays (20 Gy) induced increase in photosynthesis due to improvements in leaf water balance as indicated by increased Ψ_{leaf} under water deficits suggesting that leaves lose less water. The results support the findings of previous workers, Khodary and Moussa (2003), they reported that treatment with low dose of gamma rays (20 Gy) to dry seeds of lupine increased the total chlorophyll content, soluble sugars and photosynthetic activity. Low doses of gamma rays highly significantly increased the level of carbohydrate constituents (Nouri and Toofanian, 2001). SOD and POD are important antioxidant enzymes that detoxify active oxygen species. Treatment of soybean with gamma rays (20 Gy) was effective in increasing SOD and POD activity under drought stress. Similar findings have bean reported in *Vicia faba* by Moussa (2008), who reported that by exposing three-week-old seedlings to γ-irradiation at the dose of 20 Gy increased the antioxidant enzyme activities of SOD and POD. In the study by Wi *et al.* (2006), the induction of POD by the irradiation would be one of the defense systems activated through the ROS-mediated cellular signaling. Enhancement in peroxidase activity by radiation has also been reported by Omar

(1988) in sunflower, Sah *et al.* (1996) in barley and Stoeva (2002) in *Phaseolus vulgaris*. Meanwhile, the activities of peroxidase in radish (*Raphanus sativus*) leaves were enhanced by gamma irradiation at 10 Gy (Lee *et al.*, 2003). Our results also indicated that the plants irradiated with gamma rays (20 Gy) promoted the accumulation of osmoprotectants, such as soluble sugars, protein and proline, and decreased accumulation of MDA and electrical conductivity under drought-stress condition. Osmotic electric conductivity, soluble sugars, proline and antioxidative components are used as physiological indices of membrane stability (Reddy *et al.*, 2004). The accumulation of soluble sugars and free amino acids, including proline, protects the cell under stress by balancing the osmotic strength of the cytosol with that of the vacuole and the external environment (Kerepesi and Galibal, 2000). The results support the findings of previous workers, presowing γ-irradiation at the dose of 20 Gy can be used for increasing total protein content, total soluble sugars concentration, growth hormone (kinetin and GA_3), total yield and yield quality improvement of *Eruca vesicaria* (Moussa, 2006). Proline as a cytosolic osmoticum and a scavenger of $OH^{\cdot}$ radical can interact with cellular macromolecules such as DNA, protein and membranes and stabilize the structure and function of such macromolecules (Kavir Kishor *et al.*, 2005). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein content was obvious (Corthals *et al.*, 2000). These proteins might play a role in signal transduction, anti-oxidative defense, anti-freezing, heat shock, metal binding, anti-pathogenesis or osmolyte synthesis which were essential to a plant's function and growth (Gygi *et al.*, 1999). Anna *et al.* (2008) reported that low dose of gamma irradiation (30 Gy) enhanced protein synthesis in *Citrus sinensis*. In conclusion, applying gamma irradiation at a dose 20 Gy to soybean seeds prior to water deficit stress could partially alleviate the detrimental effect of water stress on growth through increasing photosynthesis, improving antioxidant system and promoting dry weight accumulation.

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IN VITRO PREVENTION OF BROWNING IN PLANTAIN CULTURE

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The *in vitro* propagation of plantain (*Musa paradisiaca*) is still faced with lots of challenges such as blackening or browning of tissues prior to culture due to the oxidation of phenolic compounds by the polyphenolic oxidase enzyme present in the tissue when excised. Understanding browning processes in plantain and possible ways of minimizing it during excision of explants with particular emphasis on the use of antioxidants was the purpose of this work. Tissues were surfaced sterilized with 0.1% (w/v) $HgCl_2$ for 1-6 mins to get a pure culture and then treated for two hours with different concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml of potassium citrate and citrate (K-C:C) as an antioxidant to check browning while sterile distilled water was used as control. The result showed that contamination free culture (100%) was achieved in the explants treated with $HgCl_2$ for 6 mins. Also the various concentration of K-C:C prevented browning within 2 hours before culturing the tissues thus inferring that browning in young plantain excised tissue can be greatly reduced by presoaking or pretreatment with antioxidant solution of potassium citrate-citrate before culturing them.

Key words: *in vitro*, prevention, browning, plantain, culture.

INTRODUCTION

Plantain (*Musa paradisiaca*) belongs to the Emusa section of the genus *Musa* in the family Musaceae, and is natural hybrid polyploids, diploids, triploids, or tetraploids. They are cultivated over a wide range of agro ecological zones and produce fruit all year round, contributing to their importance as staple food and valuable export commodity. It is a nutritious fruit rich in carbohydrates and a good source of iron and vitamins (Chattopadhyay *et al.*, 2000), the plant is found in several countries and continents of the tropical regions such as southeast Asia and Oceania including the modern Indonesia, Malaysia and the Philippines and northern Australia. Establishment of plantain plantation has some economical implications, which directly or indirectly stimulates agricultural and commercial ventures. This leads to accelerated developments crucial to national economic growth.

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The development of micro propagation techniques has been a major focus of Musa research during the past two decades and such technologies have now been well established (Vuylsteke, 1998; Israeli *et al.*, 1995). Plantain contain constituents of phenolic enzymes principally of polyphenoloxidase enzymes. They serve as a very important phyto auxin in plantain and help to defend the plant against infection from fungi viruses and bacteria when injured.

Phenols are chemical compounds that embrace a wide range of plant substances which possess in common an aromatic ring bearing one or more hydroxyl constituents. Phenolic substances tend to be water soluble since they most frequently occur combined with sugar as glycosides and are usually located in the cell vacuoles. Phenols are collectively called polyphenols. They are a group of chemical substances found in plant, characterized by the presence of more than one phenol unit or building block per molecule. The constituent phenols in *Musa spp* are principally doparnine, catechin, chlorogenic acid, cinnamic acid, hydroxylbenzoic, Resorcinol, progallic acid, salicylic acid, ferulic acid, vanillin, coumarin, P-coumaric acid, phenols (Khalil *et al.*, 2007). These are localized mainly in the later vessel of the pulp peep, cells and tissues disposed in the latter.

However, these phenolic compounds are actively responsible for certain browning reactions and astringency of the fruit and are responsible for high mortality rate (lethal browning) in third generation of tissue culture (Ko *et al.*, 2008).

These form a problem of in *in vitro* culture of explants, accompanied by the darkening of medium an attribute of the phenolic compound exuded from the plant tissue and accumulating in the culture medium. This browning of the surface of the explants is due to the oxidation of phenolic compounds resulting in the formation of quinines which are highly reactive to the plant tissue (Taji *et al.*, 1997).

Plantain and suckers (stem) are susceptible to tissue browning and elimination or minimization of this process are essential prerequisites to successful culture establishment. Therefore, identification of a suitable treatment to minimize tissue browning in the explants with particular emphasis on the use of antioxidants is the main objective of this work.

MATERIALS AND METHODS

Collection of Buds or Mini Suckers (meristem buds). Mini suckers of plantain were collected from the field with a machete and carried to the lab for processing. The mini suckers contain/have the buds or meristem in them. The peduncle was removed in a stepwise manner until they became too small to remove peeling by hand. Working with a dissecting scalpel and forceps, the remaining leaf/peduncle was removed and reduced to 1cm long showing the rounded growing points.

Disinfection/Sterilization Procedure. The surface sterilization procedure began with dissection of explanted material into manageable units. The auxiliary buds or meristem was treated by initially removing the small leaflets and cleaning away surface detritus under running tap water for 1 to 2 mins. A beaker (250 mls) was used for treatment with sterile solution. Sterilization was undertaken for 6 mins using 0.1% (w/v) $HgCl_2$. Explants were transferred to a separate beaker for the washing phase, in three changes of sterile distilled water (SDW) and they were cultured in culture vessels to check contamination and sterilization percentage.

Media Preparation in 1 liter Ms Medium supplemented with 20 mg/ml Ascorbic acid, 4 mg/ml BAP, 0.1 mg/ml 1AA, 100 mg/ml myo-Inositol, sugar 30 g & 2.5 g phytagel, see Table 1 below.

Table 1

Medium Composition

Composition	**M/L/g**
Micro stock × 200	5 mls
Iron stock × 200	5 mls
Vitamins stock × 200	5 mls
Ascorbic acid (20 mg/ml)	5 mls
BAP (1 mg/ml)	4.5 mls
IAA (0.1 mg/ml)	1.8 ml
Sugar	30 g
Myo-Inositol (100 g)	0.1 g
PH	5.8 (Adjust with 1 M NaOH)

The medium was stirred with a stirrer and made up to 1000 mls (IL) with distilled water using a measuring cylinder. The medium was poured into a 1 litre beaker and 2.5 g of phytagel was added. The medium was micro waved (or heated) to melt/dissolve the phytagel for 8mins. It was stirred for even distribution using a magnetic stirrer. Dispense 20 mls in culture vessel and autoclave at 121 ºC for 15min under 15 psi. Allow culture to cool and store in a cabinet to check for any growth or contamination before using.

Antioxidant Treatment. A stock solution of potassium citrate and citrate (K-C:C) was made up using 1g k-c and 0.25 g citrate and dissolved in 10 ml of SDW. The concentration was then diluted and used at a final concentration of 0.125%. The excised buds (i.e. meristem) were placed in Petri dishes containing the treatment (the antioxidant, k-C:C), 0.125% of K-C:C (as stock solution) in the concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/m1 of K-C:C to check the strength of K-C:C, *i.e.* how long and at what concentration will start or stop browning. Also the disc section was placed on filter paper and exposed to Air. It was placed in water and sterile distilled water to check browning; these served as a control. These treatments were placed in a Petri dish and results were recorded at time intervals of

0, 6, 30, 60 and 120 min. Observations of the extent of browning were recorded. The treated explants were placed in a test tube containing 20 ml of the media under a laminar flow hood and kept in the growth room to grow.

Table 2

Antioxidant Treatments

TREATMENT NUMBER	TREATMENTS	VOLUME OF ANTIOXIDANT TREATMENT (mL)
1	Expose to air, cut on wet filter for pear	0
2	Cut in Petri dish plus H_2O (SDW)	100
3	Cut in k-c: c (0.1 mg/ml)	100
4	Cut in k-c: c (0.2 mg/ml)	100
5	Cut in k-c: c (0.2 mg/ml)	100
6	Cut in k-c: c (0.3 mg/ml)	100
7	Cut in k-c: c (0.4 mg/ml)	100
8	Cut in k-c: c (0.4 mg/ml)	100
9	Cut in k-c: c (0.125/v) + L-cysteine	100
10	Cut in k-c: c (0.125) + L-cysteine+ +Ascorbic acid	100

RESULTS AND DISCUSSION

Results of the surface sterilization procedure for explants are summarized in Table 3. It showed that after a few days of culture, contamination free culture (100%) was achieved in the explants treated with $HgCl_2$ for 6 min.

Table 3

Surface Sterilization of Explant Using $HgCl_2$ Treatment

Treatment duration (min) with 0.1% $HgCl_2$	Number of explant	Percentage contamination	Percentage contamination free
1	1	100%	0
2	1	100%	0
3	1	100%	0
4	1	100%	0
5	1	100%	0
6	1	0	100%

Table 4 showed the result of the antioxidant treatment. The buds or meristem disc section from young plantain mini suckers were treated with various

concentration of antioxidant solution and a control with water, sterile distilled water (SDW) and air for duration of two hours.

Table 4

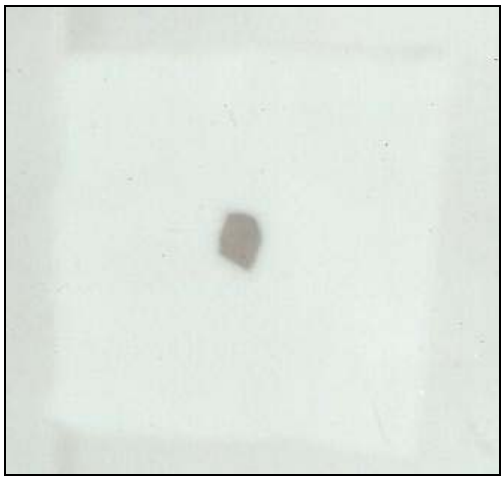
Antioxidant treatment relative browning of disc section of plant tissue treated with antioxidant for a period of two hours

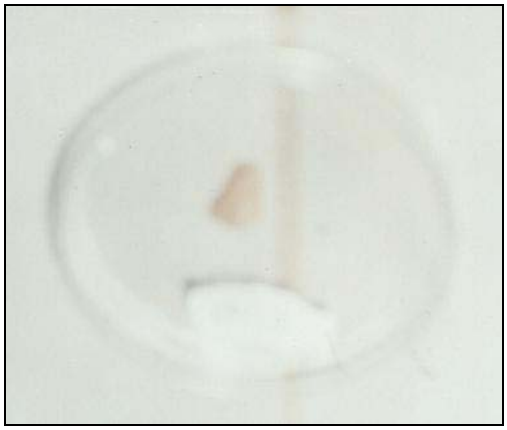
TIME (min)	TREATMENT NUMBER									
	1	2	3	4	5	6	7	8	9	10
0	+++	++	+	-	-	-	-	-	-	-
16	+++	+++	++	-	-	-	-	-	+	-
30	+++	+++	+++	-	-	-	-	-	+	+
60	+++	+++	+++	-	-	-	-	-	++	++
120	+++	+++	+++	-	-	-	-	-	+++	+++

Keys:
– = No oxidation of phenol (Thus no browning)
+ = Low oxidation
++ = Medium oxidation
+++ = High oxidation

Treatment number:
1 ⟶ Expose to air, cut on filter paper
2 ⟶ Cut in Petri dish plus H_2O (SDW)
3 ⟶ Cut in Petri dish plus H_2O
4 ⟶ Cut in K-c: c (0.1 mg/ml)
5 ⟶ Cut in K-c: c (0.2 mg/ml)
6 ⟶ Cut in K-c: c (0.3 mg/ml)
7 ⟶ Cut in K-c: c (0.4 mg/ml)
8 ⟶ Cut in K-c: c (0.5 mg/ml)
9 ⟶ Cut in K-c: c (0.125 w/v) + L-cysteine
10 ⟶ Cut in K-c: c (0.125 w/v) + L-cysteine + Ascorbic acid

a





b

Plate 1. Plantain tissue
(a) Exposed to air cut on filter paper, (b) Cut in Petri dish plus water (SDW).

Plate 2. Treated Plantain tissues.

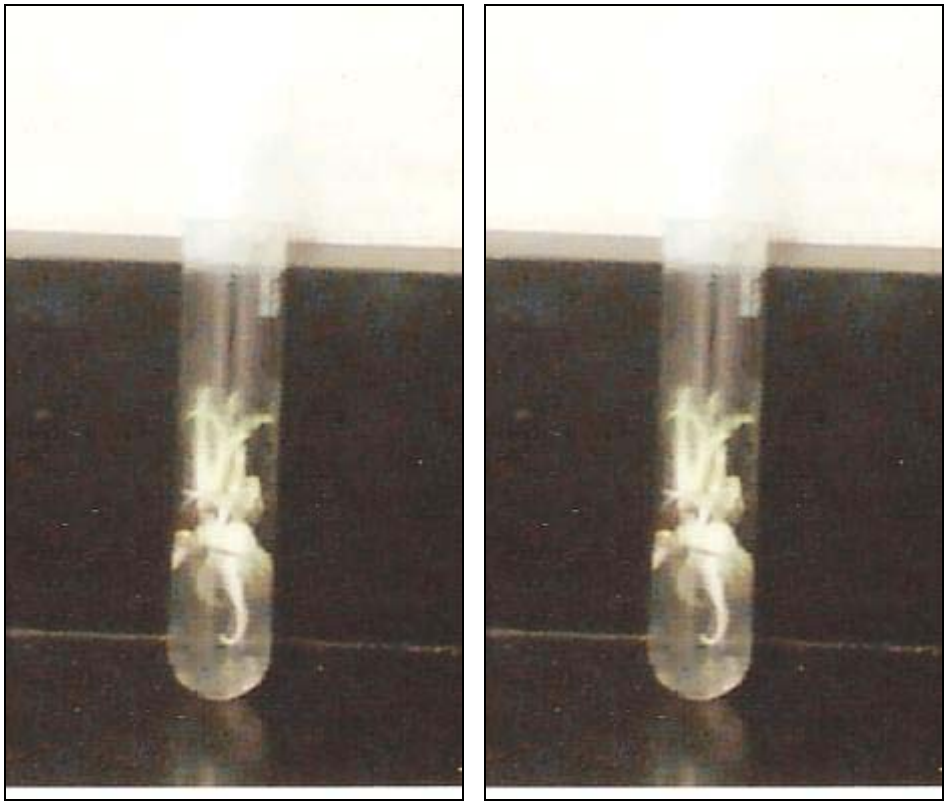


Plate 3. Plantain tissue culture showing proliferation.

The results of the treatment showed that antioxidants are electron donors (reducing agent) which inhibit the oxidation of labile substrates as described by George (1996). The antioxidant compounds utilized in the experimental work were selected because they have been used successfully in the past to delay browning in arborescent monocotyledon species (Khatri *et al.*, 1997). The disc section selected were young sucker which is highly prone to browning or oxidation of phenolic compounds which is a common problem in the establishment of plantain *in vitro* culture. All cut surfaces in the control experiment appeared to oxidize phenol rapidly once exposed to air evidenced by tissue browning. Subsequently, tissue exercised in water and SDW oxidized phenolic compounds and the tissues turned brown. The cut surface of any damaged area of untreated tissue turned brown in less than 15 minutes after excision. These explants continued to oxidize phenolic compounds and were completely brown after 2 hours and were subsequently discovered. According to Soubir *et al.* (2006), L-cysteine and ascorbic acid in combination with K-C:C initially reduced or delayed browning but oxidation of

phenolic compounds resumed after 30 min of treatment and continued the oxidation of these compounds until tissues turned brown.

Potassium citrate-citrate combination as an antioxidant treatment for excised plantain tissue proved to be the best treatment type from the treatment result (Table 2 from no. 4 to 8) and Plate 3 (a & b). The various concentration of K-C:C reduced or prevented browning within 2 hours before culturing the tissues.

The citrate in citric acid works as a chelating agent (*i.e.* it has the ability to interfere with the action of peroxidase enzymes) bonding to ions responsible for activating polyphenolic oxidative enzymes (PPO). Ascorbates behave as a reducing agent and are converted to dehydro-ascorbic acid (Panaia, 1998). Ascorbates are able to scavenge oxygen radicals produced when tissue is damaged and therefore cells are protected from oxidative injury. Oxygen radicals are attributed to exacerbating oxidative injury. These free radicals can be detoxified by antioxidants containing citrates and ascorbates, thus reducing browning of tissues (Soubir *et al.*, 2006).

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A STUDY OF PATHOGENIC FUNGI ASSOCIATED WITH CITRUS DECLINE AT THE ORCHARDS OF NIGERIA INSTITUTE OF HORTICULTURAL RESEARCH (NIHORT)

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Disease surveys were conducted at the Cirus orchards of National Horticultural Research (NIHORT), Mbato, Okigwe to ascertain pathogenic fungi associated with citrus decline. Isolations showed that *Phytophthora palmivora* (Butler), *Botryodiplodia theobromae* (Pat), *Fusarium oxysporum* (Schlechtend), and *Fusarium equiseti* (Wollen) were constantly isolated from the rhizosphere (soil) roots, stem and leaves. Pathogenicity studies confirmed these microorganisms as responsible for some of the diseases especially Citrus decline.

Key words: chemical constituents, citrus, plantation, soil, NIHORT.

INTRODUCTION

Citrus in the family *Rutaceae* of the tribe citreae have members which are fruit bearing possessing juice filled vesicle known as hesparidium.

They are thorny aromatic shrubs or small trees and shrubs about 3.5 meters in height: small trees with leathery, glossy evergreen leaves. Flowers are abundant, single, mostly white and fragrant. They are cultivated from 15°N–35°S between sea level and 1000 m. It is a tropical and subtropical crop and requires 100 cm of rainfall or else it will require irrigation (Okwulehie, 1998). Among the citrus plants are large varieties of Sweet orange (*Citrus sinensis*), Mandarin orange (*Citrus reticulate*), Grape or Pumelo (*Citrus paradise*), lemon (*Citrus limon*) and Lime (*Citrus aurantifolia*). In Nigeria local seedlings/cultivars are included. At NIHORT, various cultivars have been developed. Most species are propagated vegetatively by grafting budwood into seedling root stock of other cultivated, wild or hybrid species. The budded stock is mostly on Mandarin root stock. (NIHORT, 2003). Nigeria produces on 0.3 million tones out of the world production of 36.0 metric tones but it has the potential to produce more for both local and international markets. Annual production is 1,59200 metric tones (IITA, 2003).

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Citrus species are utilized in many industries for the production of various brands of citrus juice. Citrus juice is rich in vitamin C, folic acid and significant quantities of other vitamins, pectins and flavonoids (Anon, 1962). Citrus is one of the most important fruit tree crops in Nigeria utilized for both fresh consumption and industrial processing.

The most important bacterial and fungal diseases in Citrus orchards are generally canker, malsecco, black spot and infections caused by *Phythopthora spp.* Various other diseases such as Armallaria root rot, blast and scab are extremely serious (Timmer *et al.*, 2000). Pathogenic fungus *Diaporthe citri* has been implicated in Melanose disease in Ibadan (NIHORT, 2003).

The common symptoms of diseases observed in the field include chlorosis, branch wilt, thinning out of canopy, foot rot, brown rot, tree decline and sparse foliage, black spot, fruit tear skin and the rottening of fruits. The sustainability of citrus production is seriously threatened by increasing problems of pests diseases and weeds (IITA, 2003). At NIHORT, there has developed a massive decline in citrus structure, high level of chlorosis and shoot dieback.

Despite report of high loss in yield due to diseases in citrus crops in other countries, research findings in Nigeria have offered little basic background information. There is urgent need to revamp the Citrus industry in Nigeria in view of its numerous uses for local consumption and for export. The result of this study may assist the NIHORT management and other Citrus farmers to overcome decline in Citrus production.

The objective of this study is therefore to isolate fungi that may be associated with citrus decline, identify the fungi, and establish their pathogenicity to oranges. Some control methods will be recommended.

MATERIALS AND METHODS

Study Area. The study area is the Citrus plantation of Nigerian Institute of Horticultural Research, Mbato, Okigwe.

Sample Collection. Random samples of leaves, stems and roots of diseased sweet oranges, grape tangelo and lemon were collected at NIHORT citrus orchards at Mbato Okigwe in sterile polyethene bags for isolation and identification of pathogenic fungi at Imo State University Laboratory.

Media Preparation. The medium used was Potato Dextrose Agar (PDA) prepared according to the manufacturer's instruction. 39.6 g of powdered PDA was dissolved, made up to 1 liter with sterile distilled water and sterilized by autoclaving at 121 ^{o}C at 15 p.s.i for 15 minutes. The medium was allowed to cool before carefully pouring into 45 sterile Petri dishes. Two drops of lactic acid were added to the medium to inhibit any bacterial growth. The Petri dishes that

contained the medium were incubated for 24 hours at room temperature (28 ^{o}C) to check for sterility before use as described by Cheesbrough (2004).

Isolation of Pathogens. Diseased citrus plants (roots, stems and leaves) were randomly collected from the selected plots. They were first washed in sterile water and then disinfected with 70% alcohol. Small portions 3mm were sectioned out from the affected parts (at the margin between the rotten area and the healthy area) using scapel which was dipped into 70% alcohol and flamed before use in each round. The sections of the diseased parts were then plated into 45 petri dishes containing PDA, and inoculated at room temperature (25–28 ^{o}C) for seven days. Noticeable fungal growth was subcultured on freshly prepared culture media and similarly incubated. This was done severally to obtain pure uncontaminated isolates. Stock cultures were stored in bijour bottles at 10 ^{o}C.

Identification of isolates. Fungi isolated were characterized and identified using Barnett and Hunter (1987) and Paul *et al.* (1983). Pictures of the cultures were taken from the culture plates and from prepared slides and recorded. Percentage occurrence of fungi from plant parts (roots, stems and leaf sections) were also recorded.

Pathogenicity Studies. The species of fungi most frequently isolated and identified earlier were tested for pathogenicity by inoculating them into healthy sweet orange seedlings. 125 sweet orange seedlings were arranged in a Completely Randomized Design in 5 treatments and 5 replicates with 5 seedlings in each replicate including the control. Pure cultures of the fungi obtained from hyphal tipping were grown in PDA medium at room temperature and used to inoculate the healthy seedlings; the control seedlings were not inoculated. 125 polyethene bags were filled with sterilized soil and the seedlings transferred into the filled polyethene bags and allowed to stay for one month to be properly established before treatment. 20 ml of hyphal tip suspension of each of the isolated fungi was used to inoculate the seedlings by root dipping method respectively. Sterile water was used in the control experiments. Observations were made overtime and disease symptoms were observed and recorded.

Experimental Design. Experimental Design used was Randomized Complete Block Design (RCBD). Analysis of Variance (ANOVA) was used to separate the means using Least Significance Difference (LSD) to determine level of significance.

RESULTS

Isolation. During the isolation, the following fungi were isolated, classified, identified and confirmed to be pathogenic to the citrus seedling. They include *Phytophthora palmivora* (Butler), *Fusarium oxysporum* (Schlechtend), *Fusarium equiseti* (Corda) Sacc and *Botryodiplodia theobromae* (Pat) whose characterization

and incidence are presented in Tables 1 & 2, Figs. 1, 2, 3 and 4. These four microorganisms were constantly isolated from the various plant parts, and the soil. The following fungi *Mucor spp, Alternaria spp, Rhizoctoria sp, Botrytis spp, Aspergillus sp, Penicillium spp, Bipolaris spp* were also isolated from the various plant parts. The percentage incidence of isolates was the highest from *F. equiseti* (100%), 80% for *P. palmivora*, 80% for *F. oxysporum* and 40% for *B. theobromae* respectively Tables 2 and 3.

Pathogenicity. The symptoms observed during the pathogenicity tests included leaf yellowing, root rot, drying of leaves, shoot tip blight, thinning out of canopy, leaf curling, shoot tip dieback especially for seedlings inoculated with *P. palmivora* and the two *Fusarium spp*. Leaf chlorosis and leaf curling symptoms are peculiar with seedlings inoculated with *B. theobromae*, Table 4. These results confirm the original disease symptoms observed in the field.

Table 1

Characterization and identification of isolates

Isolates	Colony Features	Microscopic Feature	Remark
1st Isolate	Isolate/organism was seen in a culture. Growth rate was slow. Mycelium was in a white colony, delicate with purple tinge, sparse and sometimes abundant	Under the microscope, the organism was seen to have conidia of varied sizes. Microconidia borne on simple conidiophore arising laterally on the hyphae. Microconidia generally abundant, variable, oval, ellipsoid, cylindrical, straight to curved structure. Macroconidia sparse and thin walled, generally 3–5 septate and pointed at both ends. There was presence of chlamydospores	Isolate identified as *Fusarium oxysporum* Schlecht (Fig. 2)
2nd Isolate	Colony growth was rapid with dense aerial growth. Mycelium white but turned tan to brown colour with age. Underneath surface brownish	Move of macroconidia strongly septate present, sickle shaped with distinctive curvature. Apical cells move elongated in curvature and basal cell footshaped. Chlamydospores abundant and thick walled.	Isolate identified as *Fusarium equiseti* (Corda) Sacc. (Fig. 3)

Isolates	Colony Features	Microscopic Feature	Remark
3rd Isolate	Isolate seen as white dense fast growing culture, extensive in growth but gradually turned dirty white to black	Short simple conidiophore seen, conidia dark, ovoid to elongate. Mature conidia 2-celled. Intercalary chlamydspores seen	Identified as *Botryodiplodia theobromae* Pat (Fig. 4)
4th Isolate	Colony whitish culture, gradually spreading, not profuse but flat and depressed. Underneath colour is milk colour to yellowish. Aerial mycelium abundant. Colony rapid growth	Mycelium highly branched, and non-septate when young, but an old culture mycelium became septate bearing reproductive bodies (sporangia). The sporangium lemon shaped was borne symbolically on short sporangiophores. Sporangium ovoid narrow at base produced singly. Sporangia production is sparse; few chlamydospores.	Isolate identified as *Phytophthora palmivora* (Butler) (Fig. 1)
5th Isolate	Colony cream yellow. Sporangihore simple to symbolical branching, colony fast growing columella globose.	Sporangia cream yellow, zygospores brackish to brown with age	*Mucor* sp.
6th isolate	Colony black to greenish in colour. Conidiophores 1-3 septate	Conidia long and often branched chains	*Alternaria* sp.
7th Isolate	Colony richly spreading, hyaline to light grey to dark conidiophores erect, swollen heads and tree like	Conidia borne on terminally swollen heads	*Botrytis* sp.
8th Isolate	Colony grew rapidly, white to black with shades of green conidiophores unbranched, aseptate with swollen apex.	Conidia borne in chains and one celled	*Aspergillus*
9th Isolate	Colonies were fast growing, greenish in colour with some white tinges. Phialides with short neck.	Conidia one celled, in long chains	*Penicillium* sp.
10th Isolate	Mycelium hyaline, long septa, branches set off from the main hyphae at right angles.	Chlamydospores-like cells present. No conidia seen	*Rhizoctonia* sp.
11th Isolate	Mycelium dark red. Conidiophores tall and erect	Conidia single, hyaline and septate with basalscar	*Bipolaris* sp.
12th Isolate	Mycelium white with cream background. Slow spreading and flat with radiating lines from centre	Did not sporulate during the experimentation	*Unidentified*

Table 2

Percentage incidence of isolates from the plant part and the soil screened for microorganisms (Fungi)

Isolate/ Microorganisms	Root rot	Stem cancer	Leaf lesion/blight	Fruit lesion	Soil	Incidence	% incidence
P. palmivora	+	+	+	-	+	4	80
F. oxysporum	+	-	+	+	+	4	80
F. equiseti	+	+	+	+	+	5	100
B. theobromae	-	+	-	+	-	2	40

Calculations based on average of three determinations of three replications each
Legend: "+" = Presence of pathogen + = 1; "-" = Absence of pathogen - = 0

Table 3

Incidence of isolates from diseased citrus parts root, stem and leaf

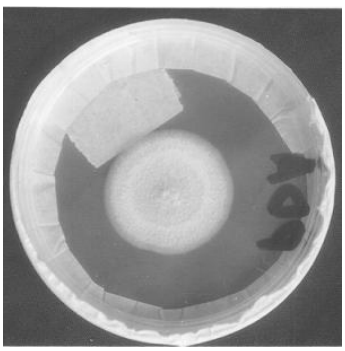
S/N	Isolates	Mean* Incidence
1	*P. palmivora*	1.75^{ab}
2	*F. oxysporum*	2.00^{ab}
3	*F. equiseti*	4.00^{a}
4	*B. theobromae*	0.50^{b}

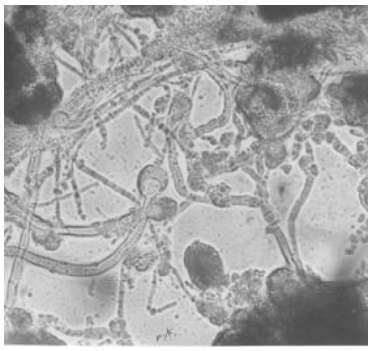
Legend "*" means with the same letter(s) are not significantly different, at p = 0.05 according to Duncan's Multiple Range Test (DMRT).

Table 4

Symptoms of disease observed during the pathogenicity test

S/N	Treatment/Isolates	Time in Months and Symptoms		
		1st & 2nd	3rd & 4th →	7th month
1	Soil sterilized and seedling inoculated with *P. palmivora*	Minor leaf mottling and yellowing of leaves observed	Mottling, yellowing and chlorosis of leaves with blight of tip of one seedling	Leaf yellowing and blight, shoot blight increase
2	Seedlings inoculated with *F. oxysporum*	Slight leaf chlorosis and yellowing	Leaf chlorosis and shoot shriveling	Shoot tip blight and deep chlorosis observed
3	Seedlings inoculation with *F. equiseti*	Minor yellowing and chlorosis of some leaves	Chlorosis and advances in shoot tip blight	Situation in months 3 & 4 deepens in some of the seedlings
4	Seedlings inoculation with *B. theobromae*	Very slight	Chlorosis appears yellowing of some leaves	Severe chlorosis in some of the seedlings


a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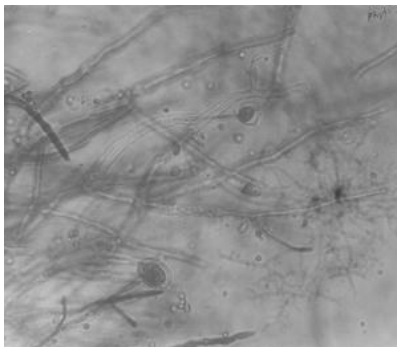

c

Fig. 1. a) Macroscopic features of *Phytophthora palmivora*,
b) Microscopic features of *P. palmivora* with sporangium,
c) Microscopic features of *P. palmivora*.


a

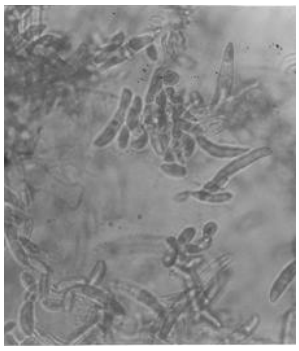
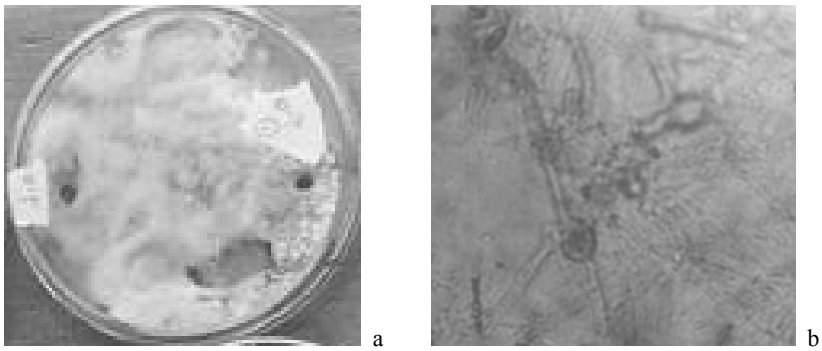
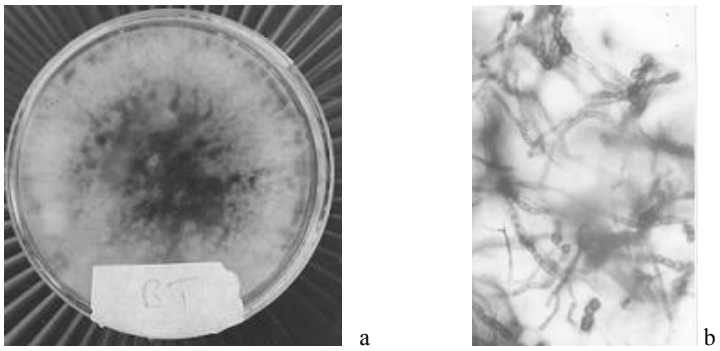

b

Fig. 2. a) Showing cultural characteristics of *Fusarium oxysporum*,
b) Showing macro and micro conidia of *Fusarium oxysporum*.



a b

Fig. 3. a) Macroscopic features of *Fusarium equiseti*,
b) Microscopic features showing conidia, chlamydospores and hyphae of *Fusarium equiseti*.



a b

Fig. 4. (a) Showing cultural characteristics of *Botryodiplodia theobromae*,
b) Microscopic features of *B. theobromae* showing conidia and chlamydospores.

DISCUSSION

Phytophthora palmivora, Fusarium oxysporum, Fusarium equisetti and *Botrydiplodia theobromae* characterized and identified in this work also caused various levels of pathogenicity to citrus seedlings as confirmed by the pathogenicity trials, through the symptoms observed. Wutscher (1998) has confirmed that *Fusarium solani* caused Fusarium twig disease of citrus. Amardor (2003) and Brlansky *et al.* (2003) have attributed dieback, root rot and gummosis diseases of citrus to the attack by various species *Phytophthora*. All these support the results of this work which indicate that the four organisms originally isolated from the diseased citrus plant parts were pathogenic to the test seedlings and may have contributed to the diseases symptoms observed in the field.

A comprehensive (integrated) approach to disease control involving biological, cultural and chemical agents should be adopted. Fungicides are better used and timely too for prevention purposes, rather than curative.

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COMPLEMENTARY EFFECTS OF *GLOMUS FASCICULATUM* AND PHOSPHATE SOLUBILIZING MICROORGANISMS ALONG WITH *RHIZOBIUM LEGUMINOSARUM* ON BLACK GRAM (*VIGNA MUNGO* L.)

K. SIVAKUMAR[1], P. THOLKAPPIAN[2]

Experiments were conducted to evaluate the effects of nitrogen fixing *Rhizobium leguminosarum*, phosphate solubilizing bacterium (*Bacillus subtilis*), phosphate solubilizing fungus (*Aspergillus awamori*) and AM fungus (*Glomus fasciculatum*) on growth, chlorophyll content, seed yield, nodulation, grain protein, and N and P uptake of black gram growing in phosphorus-deficient soils. The triple inoculation of AM fungus, *Rhizobium leguminosarum* and *B. subtilis* significantly increased dry matter yield, chlorophyll content in foliage and N and P uptake of black gram plants. Seed yield was enhanced by 24% following triple inoculation of *R. leguminosarum* + *G. fasciculatum* + *B. subtilis*, when compared to that of control. Nodule occupancy, determined by indirect enzyme linked immunosorbent assay (ELISA), ranged between 77% (*R. leguminosarum* + *A. awamori*) and 96% (*R. leguminosarum* + *G. fasciculatum* + *B. subtilis*) at flowering (45 DAS), decreasing at the pod-fill (60 DAS) stage by each treatment. Replica immunoblot assay (RIBA) revealed a greater variation in the rhizobial populations within nodules. The correlation between nodule occupancy and immunoblot counts was highly significant at 45 (r = 0.95) and at 60 DAS (r = 0.96). There was a negative effect on some of the measured parameters when *A. awamori* was used alone or added to the combination treatments. The present findings showed that rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake of black gram finally leading to improved yield.

Key words: AM fungi, ELISA, black gram, RIBA, *Glomus fasciculatum*.

INTRODUCTION

Phosphorus is one of the major nutrients limiting plant growth. Most of the soils throughout the world are with Phosphorus deficient (Batjes, 1997) and therefore require Phosphorus to replenish the P demand by crop plants. To avoid the Phosphorus deficiency in soils, Phosphorus fertilizers are applied. However, after application, a considerable amount of Phosphorus is rapidly transformed into less available forms by forming a complex with Aluminum or Ferrous in acid soils

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(Norrish and Rosser, 1983) or Calcium in calcareous soils (Lindsay *et al*., 1989) before plant roots have had a chance to absorb it. Further, the use of rock phosphate as a phosphate fertilizer and its solubilization by microbes (Kang *et al*., 2002), through the production of organic acids (Maliha *et al*., 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically (Zapata and Roy, 2004). In conjugation with phosphate solubilizing microorganisms (PSM), rock phosphate provides a cheap source of Phosphate for crop production. In this regard, several studies have conclusively shown that PSM solubilizes the fixed soil Phosphorus and applied phosphates, resulting in higher crop yields (Zaidi 1999; Gull *et al.*, 2004). The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of *Rhizobium* and PSM (Perveen *et al.*, 2002) and PSM and arbuscular mycorrhizal (AM) fungi (Zaidi *et al*., 2003) has been shown to stimulate plant growth more than inoculation of each microorganism alone in certain situations when the soil is Phosphorus deficient. AM fungi, on the other hand, encourage the plant roots to rapidly absorb solubilized Phosphorus. Accordingly the increase in plant growth may be due to the release of certain plant growth promoting substances (Kucey *et al*., 1989) by the PS organisms or AM development and mycorrhizal formation (Azcon-Aguilar and Barea, 1985). However, the inoculation effects of the tripartite interaction between N_2 fixing, PSM and AM fungus on legume crops are relatively scarce (Zaidi *et al*., 2004). Black gram (*Vigna mungo* (L.) Hepper) is a grain legume plant grown widely in the tropics and naturally fixes about 40–50 kg N ha^{-1}. In India, black gram is grown in an area of 3 × 106 ha with an annual production of 1 × 106 t of grain. Therefore, it is of great practical importance to evaluate the effect of tripartite symbioses where each partner plays a specific role in plant growth and yield. The objective of the present work was to evaluate the efficiency of the interaction between N_2 fixing *Rhizobium leguminosarum*, PS bacterium (*Bacillus subtilis*), PS fungus (*Aspergillus awamori*) and AM fungus (*Glomus fasciculatum*) on the growth, nodule occupancy, yield and Nitrogen and phosphorus uptake by black gram under non-sterilized soils, in clay pots.

MATERIALS AND METHODS

The cultures of *R. leguminosarum*, and *G. fasciculatum* were obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India. The PS bacterium, *B. subtilis* (MTCC 121) and PS fungus (*A. awamori*) were procured from the Institute of Microbial Technology, Chandigarh, India. *R. leguminosarum* was grown in yeast extract mannitol broth in flasks by shaking at 125 rpm at 28 ± 2 ˚C for 7 days to a cell density of 4 × 108 cells ml^{-1}. *B. subtilis*

and *A. awamori* were grown in National Botanical Research Institute Phosphate (NBRIP) growth medium (Nautiyal, 1999) for 6 and 3 days, respectively, at 28 ± ± 2 ˚C to a cell density of 2.3 × 108 and 4.5 × 106 cells ml^{-1}, respectively. *G. fasciculatum* was multiplied on rhodes grass (*Chloris gayana* Kunth) following the open pot culture method (Gilmore, 1968).

Seeds of black gram var. VBN (BG) 4 were surface sterilized (Vincent, 1970), rinsed 6 times with sterile water and dried. The surface disinfected seeds were coated with microorganism by soaking seeds in liquid culture medium of each organism for 2h using 10% gum arabic as adhesive to deliver 108 cells seed-1 *R.leguminosarum*, and 107 cells seed-1 *B. subtilis*. For combined inoculations, the liquid cultures of each organism were mixed in equal proportion and then seeds were dipped in it. In combined treatments with *G. fasciculatum*, the bacterized seeds were sown in soils having 100g of the mycorrhizal inoculum (infected roots and spores). Spore suspension (4 ml) of 2 × 106 ml^{-1} of *A. awamori* was added to soils 48h before sowing. The uninoculated seeds served as control for comparison. Rock phosphate (P_2O_5 23%) was added as phosphatic P (20 mg kg^{-1}) to the soil before seeding, to all treatments except control, which had 20 mg kg^{-1} N (urea) and 40 mg kg^{-1} P (single super-phosphate).

The inoculated seeds were sown in earthen pots (10 seeds pot^{-1}) having 12.5 kg of unsterilized sandy clay loam soil (alluvial, organic C 0.4%, pH 7.4, WHC 0.44 ml g^{-1}, Olsen P 16 mg kg^{-1} and Kjeldahl N 0.75 g kg^{-1}). The seeds were sown during summer of 2009 and repeated with the same treatments during summer of 2010. The pots with different treatments were arranged in a randomized complete block design with 9 replications of each treatment. The pots were kept at 22 ± 2 ° C with 60% relative humidity. Seedlings were thinned to 4 plants per pot 5 days after emergence. The plants were watered using tap water as and when required.

Treatments: T_1 – *R. leguminosarum*; T_2 – *A. awamori*; T_3 – *G. fasciculatum*; T_4 – *Bacillus subtilis*; T_5 – *R. leguminosarum*, + *A. awamori*; T_6 – – *R. leguminosarum*, + *G. fasciculatum*; T_7 – *R.leguminosarum* + *B. subtilis*;T_8 – *A. awamori* + *G. fasciculatum*; T_9 – A. awamori + *B. subtilis*; T_{10} – *G. fascic*ulatum + *B. subtilis*; T_{11} – *R. leguminosarum* + *A. awamori* + *G. fasciculatum*; T_{12} – *R. leguminosarum* + *G. fasciculatum* + *B. subtilis*; T_{13} – *A. awamori* + *B. subtilis* + *G. fasciculatum*; and T_{14} – control. The experiments were conducted during summer to ensure the reproducibility of the results. All plants in 3 pots were uprooted from each treatment at 45 (flowering stage) and 60 days (pod-fill stage) after seeding (DAS) and used for nodulation analysis. The plants were uprooted carefully and the adhering soil particles were removed by washing under water, and intact nodules were detached from roots, counted, oven dried (80 ˚C) and weighed. Nodule occupancy was determined by indirect ELISA (Kishinevsky and Bar-Joseph, 1978) while the rhizobial populations within nodules were quantified by replica immunoblot assay (RIBA) (Khan *et al*., 2002). Nodule occupancy was

calculated using the following formula and expressed in percentage = (Number of nodules positive to ELISA) / Total number of nodules tested × 100.

The rhizobial populations in each nodule was quantified as number of purple spots on the nitrocellulose membrane/ dilution factor × volume of inoculum. Plants removed at flowering (45 DAS) and harvest (80 DAS) stage were oven dried before the weights of roots and shoots and total biomass were determined. The remaining pots were maintained until harvest. Plants were finally harvested after complete maturity at 80 days after seeding and seed yield and protein contents in grain (N × 6.25) were recorded. Total chlorophyll contents in foliage were determined at 45 DAS (Mechenny, 1941). Total nitrogen content in roots, shoots and straw was measured at 80 DAS as suggested by Iswaran and Marwah (1980). Total Phosphorus contents in whole plants were estimated at 45 and 60 DAS by adopting the method of Jackson (1958). Data obtained in the present study were analysed using one way ANOVA.

RESULTS

The inoculation effects of N_2 fixing, PSM and AM fungus on black gram used either alone or in combinations were variable (Table 1). The single inoculation of *R. leguminosarum* significantly ($P < 0.05$) increased the dry matter accumulation in shoot at 45 and 80 DAS and total biomass of black gram only at 45 DAS as compared to control. In contrast, the single inoculation of *A. awamori* and *B. subtilis* significantly decreased the dry matter accumulation in root at 45 and 80 DAS, while the *G. fasciculatum* inoculation reduced the root weight only at 80 DAS. Among dual inoculation treatments, the combination of *R. leguminosarum* + *B. subtilis* significantly enhanced the dry matter accumulation in the root, shoot and dry weight of whole plant at 45 DAS and at 80 DAS as compared to control. In comparison, the co-inoculation of *G. fasciculatum* + *B. subtilis* significantly enhanced the dry matter accumulation in root and shoot at 45 and 80 DAS and total plant biomass at 80 DAS only as compared to the control. Addition of *G. fasciculatum* to *R. leguminosarum* + *B. subtilis* resulted in a significant ($P < 0.05$) increase in the dry matter of root and shoot and total plant biomass of black gram at both 45 DAS and 80 DAS as compared to control. In contrast, *A. awamori* + *B. subtilis* + *G. fasciculatum* significantly enhanced the dry matter accumulation in root at 45 and 80 DAS, the dry matter accumulation in shoot at 80 DAS, and total biomass at 45 DAS, when compared to control. The triple inoculation of *R. leguminosarum* + *G. fasciculatum* + *B. subtilis* augmented the total dry weight of black gram at 80 DAS by 165% as compared to control. It was superior to all other treatments. Seed mass in general increased significantly with all the treatments except T_2 and T_4 or combined inoculation where *A. awamori* was

included, relative to the control. Seed yield increased by 14% and 11% due to inoculation with *R. leguminosarum* and *G. fasciculatum*, when used alone, respectively. Seed yield increased even further, by 15% and 17%, respectively, when *R. leguminosarum* + *G. fasciculatum* and *G. fasciculatum* + *B. subtilis* were used together, over the control. However, seed mass declined by 36% and 9% with T_4 and T_2 inoculation, respectively, compared to the control. The efficiency of *R. leguminosarum* was more pronounced when it was applied in combination with *G. fasciculatum* + *B. subtilis* and increased seed yield by 24%, which was followed by a 21% increase with the triple inoculation of *A. awamori* + *B. subtilis* + + *G. fasciculatum* (T_{13}). The total biomass and seed yield were positively correlated (r = 0.4). Chlorophyll contents increased significantly at the flowering (45 DAS) stage with *R. leguminosarum* alone, and composite cultures of *Rhizobium leguminosarum* + *B. subtilis* and *G. fasciculatum* + *B. subtilis*. The chlorophyll content following the application of three organisms together, T_{13} and T_{12} recorded 52% and 86%, respectively, were higher than control (Table 1).

Nodulation response to experimental treatments under non-sterilized pot soil was highly variable (Table 2). A significantly ($P < 0.05$) greater number of nodules per plant of black gram plants inoculated with *Rhizobium leguminosarum* alone, and the dual and triple inoculation treatments (except for T_9 and T_{10}) both at 45 and 60 DAS, compared to the control. Among the dual inoculation treatments, *R. leguminosarum* + *G. fasciculatum* had the greatest positive effect on the number of nodules at the flowering (45 DAS) and pod-fill (60 DAS) stages as compared to control and showed an increase of 7% and 6% at the flowering and pod-fill stages, respectively, over T_1. A significantly higher number of nodules was recorded in triple inoculations of *R. leguminosarum* + *G. fasciculatum* + *B. subtilis* as compared to T_1 at 45 DAS (48 nodules/plant). Inoculation of *A. awamori* with *R. leguminosarum* or *R. leguminosarum* + *B. subtilis* significantly ($P < 0.05$) increased the number of nodules at both stages of plant growth as compared to control but the number of nodules was considerably decreased when compared to that of T_1. Generally, the number of nodules was more at 45 DAS as compared to 60 DAS in all treatments. Nodule occupancy as determined by indirect ELISA ranged between 77% (*R. leguminosarum* + *A. awamori*) and 96% (*R. leguminosarum* + *G. fasciculatum* + *B. subtilis*) at 45 DAS, while it was between 63% (T_{11}) and 96% (T_{12}) at 60 DAS. Nodules collected from treatments not inoculated with *R. leguminosarum*, however, showed no positive immunoreaction in the ELISA test. Moreover, the host plant differed significantly with regard to their nodule dry weight, and the correlation between number of nodules and its dry mass was highly significant at 45 DAS (r = 0.99) and at 60 DAS (r = 0.95). The establishment and survival of *R. leguminosarum* in nodules as determined by RIBA test varied considerably (Table 2).

Table 1

Co-inoculation effect of nitrogen fixing and phosphate solubilising microorganisms and AM fungus on growth, seed yield and chlorophyll content of black gram

Treatments	Mean dry mass (g plant^{-1})				Total dry mass (g plant^{-1})		Seed mass (g 1000 seed^{-1})	Chlorophyll (mg plant^{-1})
	Root		Shoot					
	45 DAS	80 DAS	45 DAS	80 DAS	45 DAS	80 DAS		
T_1 *Rhizobium leguminosarum*	0.5	0.7	1.5	1.8	2.0	2.5	34.5	2.7
T_2 *Aspergillus awamori*	0.3	0.4	0.8	1.1	1.1	1.5	27.6	1.8
T_3 *Glomus fasciculatum*	0.6	0.6	0.8	0.9	1.4	1.5	33.6	2.1
T_4 *Bacillus subtilis*	0.4	0.6	0.7	0.9	1.1	1.5	19.2	2.1
T_5 *R. leguminosarum* + *A. awamori*	0.5	0.6	0.8	0.9	1.3	1.5	25.4	1.8
T_6 *R. leguminosarum* + *G. fasciculatum*	0.9	1.1	1.1	1.4	2.0	2.5	34.8	1.6
T_7 *R.leguminosarum* + *B. subtilis*	0.3	1.3	1.5	1.8	1.8	3.1	32.2	2.9
T_8 *A. awamori* + *G. fasciculatum*	0.4	0.8	1.4	1.8	1.8	2.6	24.4	2.0
T_9 *A. awamori* + *B. subtilis*	0.4	0.7	0.8	1.4	1.2	2.1	26.6	1.9
T_{10} *G. fasciculatum* + *B. subtilis*	0.8	1.3	1.7	2.3	1.5	3.6	35.2	3.2
T_{11} *R. leguminosarum* + *A. awamori* + *G. fasciculatum*	0.7	1.2	1.5	2.5	2.2	3.8	30.2	3.1
T_{12} *R. leguminosarum* + *G. fasciculatum* + *B. subtilis*	1.5	1.8	2.4	3.5	3.9	5.3	37.4	3.9
T_{13} *A. awamori* + *B. subtilis* + *G. fasciculatum*	0.9	1.4	0.9	1.5	1.8	2.9	36.6	3.2
T_{14} control	0.6	0.8	0.8	1.2	1.4	2.0	30.2	2.1
LSD (P = 0.05)	0.12	0.18	0.21	0.25	0.36	1.02	1.3	0.52

Table 2

Co-inoculation effect of nitrogen fixing and phosphate solubilising microorganisms and AM fungus on nodulation, immunoblot counts and grain protein, and N and P content in black gram

Treatment	Nodule						Immunoblot counts ($\times 10^5$ cells ml^{-1})		Grain protein (%)	N content (mg $plant^{-1}$)			P content (mg $plant^{-1}$)	
	Number of nodules plant		Occupancy (%)		Dry mass (mg $plant^{-1}$)									
	45d	60d	45d	60d	45d	60d	45d	60d		root	shoot	straw	45d	60d
T_1 *Rhizobium leguminosarum*	41	34	86	75	18.6	12.8	112	90	25.2	16.4	30.5	20.4	1.2	2.4
T_2 *Aspergillus awamori*	-	-	-	-	-	-	-	-	15.5	11.1	19.6	21.2	1.3	2.4
T_3 *Glomus fasciculatum*	4	3	-	-	3.6	3.2	-	-	20.4	10.7	14.4	12.8	1.2	2.2
T_4 *Bacillus subtilis*	5	4	-	-	2.8	2.5	-	-	17.4	11.2	15.0	17.5	1.4	2.6
T_5 *R. leguminosarum* + *A. awamori*	34	23	77	68	13.4	12.4	127	120	21.5	16.8	32.8	26.6	1.6	3.1
T_6 *R. leguminosarum* + *G. fasciculatum*	46	36	87	86	20.3	20.5	131	110	26.7	19.4	33.4	28.4	1.8	3.1
T_7 *R. leguminosarum* + *B. subtilis*	44	23	93	89	21.2	18.2	126	120	25.2	19.8	40.2	29.6	1.7	3.4
T_8 *A. awamori* + *G. fasciculatum*	-	-	-	-	-	-	-	-	11.4	15.4	31.6	28.5	1.8	2.9
T_9 *A. awamori* + *B. subtilis*	5	3	-	-	3.6	3.3	-	-	19.8	17.6	19.4	12.4	2.6	4.2
T_{10} *G. fasciculatum* + *B. subtilis*	5	4	-	-	3.2	3.0	-	-	22.0	19.7	20.5	22.6	2.2	3.8
T11 *R. leguminosarum* + *A. awamori* + G. fasciculatum	31	22	83	63	12.5	10.4	66	57	25.4	20.7	26.0	20.6	2.5	3.9
T12 *R. leguminosarum* + *G. fasciculatum* + *B. subtilis*	48	31	96	96	19.8	18.4	156	145	28.6	26.8	30.3	27.2	2.8	4.2
T13 *A. awamori* + *B. subtilis* + *G. fasciculatum*	-	-	-	-	-	-	-	-	26.5	19.0	25.0	22.2	2.8	4.3
T14 control	6	5	-	-	3.6	3.4	-	-	20.1	17.2	20.8	19.6	1.2	2.3
LSD (P = 0.05)	4.1	3.7	11.5	11.4	2.7	2.6	Nd	Nd	4.4	3.8	5.8	4.9	0.3	0.6

The populations of *R. leguminosarum* within nodules were significantly higher at 45 DAS (156 × 105 cells ml^{-1}) and 60 DAS (145 × 105 cells ml^{-1}) in the nodular suspension prepared from nodules of the triple inoculation treatment (*R. leguminosarum* + *G. fasciculatum* + *B. subtilis*). The nodular suspension of *R.leguminosarum* + *A. awamori* + *G. fasciculatum* treatment, however, revealed the lowest rhizobial counts at 45 DAS (66 × 105 cells ml^{-1}) and 60 DAS (57 × 105 cells ml^{-1}). The nodule occupancy and immunoblot counts were highly correlated at both 45 DAS (r = 0.95) and 60 DAS (r = 0.96). A maximum increase of 42% in grain protein was found with *R. leguminosarum* + *G. fasciculatum* + *B. subtilis*, which was followed by a 33% increase due to dual inoculation of *R. leguminosarum* + *G. fasciculatum* over control. *A. awamori*, either alone or in dual inoculation treatments, however, depressed the seed mass and grain protein. Single inoculation of *A. awamori* and combined inoculation of *A. awamori* + *G. fasciculatum* decreased the GP significantly by 23% and 43%, respectively, as compared to control. Single inoculation treatments in general (except for T_1) depressed the nitrogen content in all parts of plants, when compared to that of control.

In contrast, the dual inoculations (except for T_9 and T_{10}) significantly stimulated the nitrogen content in the shoot and straw of black gram as compared to control. Among the dual inoculations, the composite application of *R. leguminosarum* + *B. subtilis* augmented the nitrogen content in the root, shoot and straw by 15%, 93% and 51% respectively over the control, and 33% and 9% in the shoot and straw respectively over T_{12} (Table 2). The triple inoculation of *R. leguminosarum* + *G. fasciculatum* + *B. subtilis* (T_{12}) enhanced the nitrogen content in root, shoot, and straw of black gram by 29%, 17% and 34%, respectively, as compared to T_{11}, and 41%, 21% and 23% over T_{13}. The increase in plant biomass was positively correlated with the nitrogen content of the root (r = = 0.8), shoot (r = 0.5) and straw (r = 0.2). The single inoculation treatments, in general, did not have any significant effect on phosphorus content of black gram at 45 and 60 DAS as compared to the control. In comparison, the dual and triple inoculation treatments significantly ($P < 0.05$) improved phosphorus content at both 45 and 60 DAS, over control. Among the dual inoculation treatments, the co-inoculation of *A. awamori* + *B. subtilis* (T_9) showed the greatest positive effect on phosphorus content and increased the phosphorus content by 117% and 83% at 45 and 60 DAS, respectively, over control. The addition of *G. fasciculatum* to the combination of *A. awamori* + *B. subtilis* further improved the phosphorus content by 133% and 83% at 45 and 60 DAS, respectively, over T_{14}, and a marginal increase over the best performing pairing of T_9. The effect of microbial inoculation on total biomass and phosphorus uptake was positively correlated.

DISCUSSION

The increased nodulation, N_2 fixation and yield of legume crops following inoculation with N2 fixing and Phosphate solubilizing microorganisms have been reported by Gupta, 2004. Presently, rock phosphate is being chiefly employed to sustain soil phosphorus level in an available form for plants. In this context, PSM have been reported to solubilize the rock phosphate through the production of organic acids, ion chelation and exchange reaction in the growth environment (Yadav and Dadarwal, 1997). As a result of this activity, PSM play an important role in supplementing phosphorus to plants, allowing a sustainable use of phosphatic fertilizers. In the present study, the addition of rock phosphate along with microbial cultures greatly enhanced the plant growth, symbiosis and nutrient uptake of black gram. It is generally thought that PSM in addition to solubilizing inorganic phosphorus also release growth-promoting substances (Kucey *et al.*, 1989), which improve germination and growth of plants and stimulate microbial activity in the rhizosphere. The present study thus clearly indicated that rock phosphate when used along with microbial treatments (*A. awamori* and *B. subtilis*) was transformed into available forms of phosphorus, as indicated by the increased phosphorus in plants and then it uptakes by growing plants and consequently enhanced the overall growth of black gram. In some co-inoculation treatments, the growth of roots and shoots was poorly stimulated, suggesting the inadequate solubilization of rock phosphate by PSM and consequently the poor availability of phosphorus to plants.

However, the tripartite cultures, in general, were significantly effective as compared to other treatments. The explanation of this fact is that the mycorrhizal endophyte could be stimulated in quantity, efficiency and longevity by metabolic product released from the inoculated bacteria. Moreover, root exudation and plasticity might have been changed by PSM inoculation, which could also affect AM development (Poi *et al*., 1989). Further, the N and P contents in plants were increased in the present study, which in turn positively and synergistically affected the development of black gram (Table 2). Generally, the addition of *A. awamori* to the N_2 fixer, AM fungus or both was either inferior or negatively affected the parameters studied. The result of this relationship could be due to the negative interaction that may have occurred between PS fungus and nodule bacteria or AM fungus (Zaidi *et al*., 2004). The resulting inhibitory effect of *A. awamori* on the associative partner could be due to the release of inhibitory metabolites in the growing environment, which in turn adversely affected the plant growth. Furthermore, the P-releasing fungi produce more organic acids (Venkateswarlu *et al*., 1984) than bacteria, which enhance the solubilization of phosphate. However, most rhizobia prefer neutral or alkaline conditions for the establishment of a functional symbiosis and therefore the increased acidity might have changed the microenvironment, which possibly decreased the survival of nodule bacterium

or colonization of AM fungus in the black gram rhizosphere. Seed bacterization temporarily changes the balance of the rhizosphere populations and such changes may sometimes enhance the plant growth, yield and uptake of nutrients depending upon the establishment of the introduced cultures. Accordingly, *B. subtilis, R. leguminosarum*, and AM fungus used in the present study were good competitors since growth, nutrient uptake and yield of black gram in the present study increased to a greater extent. The fact that plant growth and nutrient uptake increased in the presence of AM fungi suggested a strong synergistic relationship between root colonization, P uptake and growth promotion. In agreement with these findings, Zaidi *et al*. (2003) observed that in low P soils plant growth and nutrient uptake in chickpea were greater after inoculation with tripartite culture of Mesorhizobium, PSB and *G. fasciculatum* than after inoculation with each organism alone. The number of nodules produced on legume plants has generally used an index for assessing the N_2 fixing efficiency of nodule bacteria. However, this does not reflect the true efficiency of particular rhizobial strains since manually counted nodules may also include nodules produced by the indigenous populations. Therefore, special attention was paid to establish the fact that the nodules produced on the root system of black gram were produced only by the introduced rhizobia. Surprisingly, nodules formed on the root systems of black gram that were even not inoculated with *R. leguminosarum* (*e.g.*, control and some other treatments). Such nodules were therefore also subjected to indirect ELISA analysis in order to ensure the specificity of nodules (Table 2). During this study, none of the nodules was collected from any of the treatments, excepting those inoculated with *R. leguminosarum*, which were positive to homologous antisera in the ELISA test. This finding suggested that nodules produced on the roots of treatments other than *R. leguminosarum* were produced by indigenous rhizobia. All the nodules collected from *R. leguminosarum* inoculated plants, however, showed 100% immuno-reactivity with their homologous antisera in the ELISA test. The better nodulation in the case of composite inoculation at the flowering stage appeared to be a result of the favorable effects of PSM in making P more soluble and available to the plants, which consequently promoted root development. In the present study, a positive correlation between the biomass and nodule numbers and N and P contents of black gram further suggested the involvement of N and P in the establishment of an effective *R. leguminosarum* – black gram symbiosis, which consequently increased the biological N_2 fixation, and yield of black gram. Similar evidence on the effect of P in N_2 fixation in French bean is reported by Saber *et al*., 2005 and in wheat by Zaidi and Khan, 2005.

In the present study, the establishment and survival of *R. leguminosarum* in the nodules were determined using a most sensitive and rapid serological method, RIBA. The nodular suspension of each treatment when tested individually with the homologous antisera produced 84%-98% purple spots on the nitrocellulose membrane in the RIBA test, indicating a greater degree of rhizobia specificity in

the nodules of inoculated plants (Table 2). Interestingly, nodules produced on the root system of black gram inoculated either with *R. leguminosarum* alone or treatments receiving *R. leguminosarum* showed 2%-16% serologically unrelated (white) spots on the nitrocellulose membrane, suggesting the appearance of an indigenous rhizobial population within a single nodule. Galiana *et al*., 1994; Khan *et al*., 1999 have also reported such rhizobial diversity in the nodules. The nodule occupancy and immunoblot counts of rhizobial populations within nodules thus provided strong evidence of an effective symbiosis, evident from the correlation between nodule occupancy and immunoblot counts at 45 (r = 0.95) and at 60 DAS (r = 0.96).

CONCLUSION

In conclusion, this study revealed that the mixed inoculation of N_2 fixing bacterium, PSM and AM fungus improved plant vigor and nutrient uptake and dramatically increased the yield of black gram in unsterilized soil. Further, the combination of *R. leguminosarum* + *B. subtilis* + *G. fasciculatum* was more effective than other single, dual or triple inoculation treatments. This combination along with rock phosphate can be used for increasing the yield of black gram, concomitantly saving considerable amounts of N and P fertilizers, and it can also be used under field conditions.

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