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Virus Elimination from Infected Garlic Plants Using Different Techniques

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Production of garlic virus free plant was attempted by means of chemotherapy, meristem-tip culture and thermotherapy techniques. Three different meristem sizes (5, 3 and 1 mm long) were excised from three garlic cultivars (Chinese, Italian and Balady). Meristems were cultured on MS medium amended with 0.5 mg/L BA (Benzyle adenine). The best size for virus elimination and survival plants was 3 mm long whereas 5 mm long produced highest survival plants without virus elimination and 1 mm long failed to produce survival plants in two of the tested three culitvars. The suitable medium was MS + 0.5 mg/L BA that produced 33.3% survival plantlets whereas MS + 0.1 mg/L NAA + 0.5 mg/L BA and MS + 0.1 mg/L NAA produced 16.6 and 6.6% survival plantlets, respectively. Virazole (50 mg/L) was used as a virus inhibitor material in MS medium for culturing meristems (3 mm long) of cvs. Chinese and Balady. The produced plantlets were 100% virus free. Garlic cloves of two cultivars Balady and Chinese were treated with hot water and hot air at different terminated degrees of temperatures and periods. Treated cloves were planted in pots.

INTRODUCTION

In Egypt garlic is considered one of the main vegetable crops, it is not only used as a food but also is important from the medical point of view. It is grown commercially in most parts of the country as winter crop.* The cultivated area has decreased from 25374 feddans in 1996 to 16632 feddans in 1997 producing 255488 and 159111 ton, respectively. The most common varieties cultivated in Egypt are the Balady and followed by the Chinese.

Graichen *et al.* (1985) reported that 15 garlic cultivars from 8 sources were examined by biological, serological (ELISA) and EM methods. All plants of 14 cultivars from all sources were infected with *Garlic mosaic virus* (GMV) and *Garlic latent virus* (GLV).

Garlic is subjected to numerous viruses such as *Garlic mosaic virus* (La, 1973 and Dijh *et al.*, 1993), *Garlic yellow streak virus* (Mohamed and Young, 1981 and Dijh *et al.*, 1993) and *Onion yellow dwarf virus* (El-Kewey and Sidaros, 1996).

Cultivated garlic is vegetatively propagated for commercial production because it is sexually sterile; therefore improvement of garlic through classical breeding techniques is not possible. Vegetative reproduction of this crop has resulted in low propagation rates and the transmission of virus disease, for this reason *in vitro* techniques have been developed for garlic production (Nagasawa and Finer 1988 a and b). Further more; tissue culture technique is a quick method for production of large numbers of virus-free plants.

*Ministry of Agric., repport of Economic Agric. sector (1997).

The purpose of this study was to:

1- Produce virus free plants of different garlic cultivars using meristem culture technique.
2- Eliminate garlic viruses from diseased garlic by chemotherapy and thermotherapy.

MATERIALS AND METHODS

The following methods were carried out to produce virus free garlic plantlets.

Tissue culture technique

The following steps and experiments were carried out:

1.Sterilization and tip meristem isolation:

Mature cloves of stored virus infected-garlic bulbs of two cultivars (Balady and Chinese) were surface sterilized inside the culture cabinet Laminar flow hood (Farma Scientific – U.S.A model 1840 s/N1535 Amprting Ncludes outlet).

Cloves sterilization were carried out as the followed steps:

1- Cloves were immersed in70% ethanol for 8-10 minutes.
2- Transferred to solution of 5.25% of sodium hypochloride or 20% commercial Clorox.
3- Rinsed three times with autoclaved distilled water.

Meristems of garlic cloves are situated at the center of the basal stem disc, on upper surface. The shoot apex is left within and at the bottom of the youngest tubular leaf (Jones and Mann, 1963).

Under aseptic condition in a sterile laminar flow Hood, using sterilized scalpel, bud forceps and needles which sterilized repeatedly between and during the operation processes of meristems excision by dipping them in 70% ethanol and flaming (Stace-Smith and Mellor 1970), the storage leaf was removed from each clove, then the tip – meristem with at least one primordium leaf was cut in three size (1 mm, 3 mm and 5 mm) in length for culturing on Murashige and Skoog (1962) medium (MS) with or without supplements.

2. Culture media:

Explants (excised tip – meristems) were cultured on MS as a basal medium (From Sigma) containing 0.8 % difeco agar, 10% sucrose.

The medium was distributed into autoclavble plastic jars (40 mL/each) or culturing tubes (15 mL/each). then they autoclaved at 121 °C for 15 min. After autoclaving both jars and tubes were transferred into the laminar flow hood.

3 culturing process:

Garlic meristem–tips (Explants) excision and culturing steps were carried out inside a sterilized laminar flow.

Excised meristems were cultured on the surface of the media either in jars or tubes. The container was directly plugged with framed caps and sealed with parafilm.

4. Incubation:

Cultivated meristems were incubated in growth Chamber (percival–MFO. CO. Boone. Iowa Model1-35LLvL.). Culture conditions were 16:8 light/dark photo period with light intensity of 1000 lux and temperature was maintained at 26 C. Meristems were subcultured onto fresh media every three weeks.

5. Tip – meristem experiments:

Three experiments were carried out under tip – meristem title, to determine the optimum size of tip

meristem, the best medium and the optimum culturing date of garlic growth, shooting, rooting, survival and virus elimination.

1- Three different sizes 1, 3 and 5 mm long of tip meristem were cut from the three cultivars (Chinese, Italian and Balady) to determine the optimum tip size for virus elimination. The culturing medium was MS supplemented with BA.

2- Three different media MS +0.1 mg/L NAA, MS +0.5 mg/L BA and MS +0.1mg/L NAA +0.5 mg/L BA, were used to determine the best medium for garlic growth, shooting, rooting and survival plants. Tip meristems of garlic cv. Chinese with 3 mm in long were cultured on the tested media.

3- The harvested garlic bulbs were stored one month at 10 °C to break dormancy of the cloves, then three different dates for tip meristem excisting were carried out at August, october and December 1995. Tip meristems of cv. Balady with 3mm long were cultured on MS +0.5 mg/L BA medium.

B. Chemotherapy

Chemotherapy of *in vitro* garlic plantlets for garlic mosaic disease elimination experiment was conducted using virazole. Tip meristem 3 mm length of two garlic cultivars, Chinese and Balady were cultured on MS medium for 10 days, then the succeeded explants transferred to MS medium amended with 50 mg/L virazole (MSV) for 6 weeks then shoots of 0.1-0.2 cm were rexcised and cultured on MS medium according to AVRDC (1994).

Data were taken 30 days after culturing of explants as follows:

1. Percentage of survival plants.
2. Percentage of survival explants that produced plantlets virus-free using *Ch. amarnticolor* as an assay host.

C. Thermotherapy

Garlic cloves of two cultivars, Balady and Chines were treated with hot water and hot air at different terminated degrees of temperature and also terminated periods. Treatments with hot air were at 35, 40, 45, 50, 55and 60 °C. Periods were 30, 40, 50, 60, 75, 90 minutes for each treatment. and with hot water were at 40, 45, 50, and 55 °C, Periods were, 25, 30, 45, 60, 75 minutes for each treatment. Cloves of both cultivars Chinese and Balady were planted in pots, kept in greenhouse to determine percentage of survival and germinated cloves, and virus free plants. Symptomless plants were tested by inoculation on *Ch. amaranticolor*.

RESULTS AND DISCUSSION

Virus like symptoms were observed on most of garlic plants in the field in Kafr El-Sheikh Governorate. Intensive studies were carried out to produce virus free plants of different garlic cultivars using meristem culture technique and eliminate garlic viruses from mosaic diseased garlic by chemotherapy and thermotherapy.

A. Meristem tip culture technique

1. Meristem size:

Data presented in Table (1) indicate that the highest percentage of survival plantlets (72.7%) was obtained when 5 mm meristem size of cv. Chinese was cultured *in vitro* on Murashige-Skoog medium (MS) amended with 0.5 mg/l BA, but at the same time, this size failed to produce virus free plantlets. The optimum meristem size which produced 40%

survival and 33.3% virus free plantlets was 3 mm long of cv. Chinese. The same size 3mm long of cv. Italian produce 46.66 % survival and 28.57 % virus free plantlets, whereas the percentage of survival plantlets of cv. Balady decreased to 3.3 % and failed to produce virus free plantlets.

2. Culture media:

Results obtained in Table (2) showed that the highest percentage of survival plantlets 33.33% was obtained when 3 mm meristem size of cv. Chinese was cultured on MS medium amended with 0.5 mg/L BA (Fig. 1), whereas MS +0.1 mg NAA/L and Ms +0.1 mg NAA/L + 0.5 mg/L BA were used, the percentage of survival plantlets were 6.6% and 16.6 %, respectively.

3. Different culture dates:

In preliminary experiments, specially in the case of cv. Balady, it was difficult to obtain reasonable percentage of survival plantlets from new yield of garlic bulbs after one month of storage at 10 °C to break dormancy of garlic cloves.

The present experiment was carried out to study the suitable time for existing meristem after storage period to be used for tissue culture experiments.

The presented data in Table (3) revealed that the highest percentage of survival plantlets (18.86%) of cv. Balady was obtained when 3 mm meristem size of cv. Balady was excised and cultured in December, whereas the percentage of survival plantlets was 3.33% and 9.52 % when cultured in August and October, respectively.

Table (1): Effect of meristem size on percentage of surviving and virus free plants of the three garlic cultivars.

Cultivar	Size of Meristems (mm)	No. of existing meristems	No. of Surviving Plantlets	Percentage of surviving Plantlest	No. of virus free plants	ercentage of virus free plants
Chinese	1	28	6	21.42	3	50.00
	3	42	18	40.00	6	33.33
	5	22	16	72.72	0	0.00
Italian	1	12	0	0.00	0	0.00
	3	30	14	46.66	4	28.57
	5	3	1	33.33	0	0.00
Balady	1	20	0	0.00	0	0
	3	60	2	3.33	0	0
	5	10	0	0.00	0	0

Table (2): Effect of media on percentage of survival garlic plantlets cv. Chinese.

Media	No. of existing meristems	No. of surviving plantlets	Percentage of Survial plantlets
MS+ 0.1 mg/L NAA	30	2	6.6
MS+ 0.5 mg/L BA.	30	10	33.33
MS+ 0.1 mg/L NAA+0.5mg/L BA	30	5	16.6

Table (3): Effect of different culturing dates of cv. Balady meristems on percentage of survival garlic plantlest.

Storage period (month)	No. of existing meristems	No. of surviving plantlets	Percentage of Survival plantlets
0*	60	2	3.33
2	63	6	9.52
4	106	20	18.86

* After dormancy break.

Meristem tip culture in garlic has been reported by Havranek (1973), Hussey (1978), Walkey *et al.* (1984), Lin (1985), Bertaccini *et al.* (1986), Novak *et al.* (1986), Lee *et al.* (1988), Chovelon *et al.* (1992), Wei and Wu (1992), Peiwen *et al.* (1993), Cardenas *et al.* (1994), Taha (1995) and Verbeek *et al.* (1995).

B.Chemotherapy of *in vitro* garlic plantlets

Data represented in Table (4) revealed that the highest percent of virus free plants (100%) for both cultivars was obtained by culturing meristems on MS medium amended with 50 mg/L virazole.

Adding chemicals with anti-viral properties to plant tissue culture medium and excising shoot tips has in the case of the synthetic riboside ribavirin (virazole) resulted in elimination of three of the major potato viruses X, S and Y (Klein and Livingston, 1982 and Wambbugu *et al.*, 1985).

Klein and Livingston (1983) reported that ribavirin treatment of cultured potato shoot tips was tested as a mean of eradicating PVX and PVS. Virus assays indicated that 93 and 87 % of the plantlets were free of PVX and PVS respectively, after treatment with 10 µg/ml medium and 20 µg/ml resulted 100 % eradication of PVX and PVS.

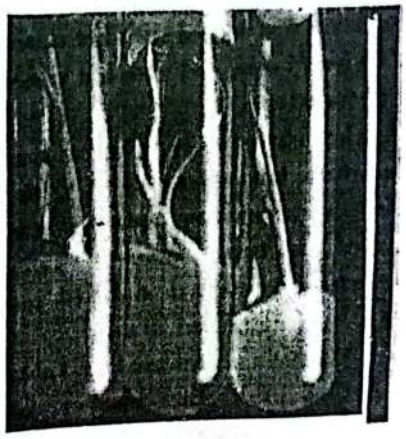

Fig (1). Meristems of three garlic cultivars: Balady (right), Italian (middle) and Chinese (left) after culturing on MS +0.5 mg/LBA.

The system appears to be efficient for monitoring the effects of antiviral treatment (ribavirin) that appear to selective inhibit viral replication before phytotoxic levels are reached. The level of ribavirin used in this study (50 mg/L) did not appear to inhibit plantlet growth. This result is in harmony with Bittner *et al.* (1989) and Slack *et al.* (1990).

Omar *et al* (1993) used chemical therapy (ribavirin) to eliminate PVY of *in vitro* plantlets.

C. Thermotherapy

For Garlic mosaic virus es elimination, cloves of two cultivars (Chinese and Balady) were treated with hot water and hot air at different degrees of temperature for different periods.

C.1. Using hot water:

Results demonstrated in Table (5) indicate:

1-When cloves of cv. Chinese were treated with hot water (40 C0) for different periods ranged from 25 to 75 min the number of surviving plants was higher than other treatments, but no virus free plants were obtained and all plants were infected.

2-Only one plant was virus free, when cloves was treated with hot water (45 °C) for 75 min. and with hot water (50 °C) for 60 min.

3-When cloves of cv. Chinese was treated with hot water (55 °C), the number of surviving plants was decreased and two plants were virus free when cloves were treated with hot water (55 °C) for 25 and 30 min.

Also data represented in Table (5) showed that the number of surviving plants was higher than other treatments when cloves of cv. Balady were treated with hot water (40-45 °C), but no virus free plants were obtained. Only one virus free plant was found when cloves were treated with hot water (50 °C) for 60 min., and two virus free plants was obtained when cloves were treated with hot water (55 °C) for 30 min.

C.2. Using hot air:

The obtained results (Table 6) showed the followings:

1-When cloves of cv. Chines were exposed to hot air (35-45 °C) for different periods ranged from 30 to 90 min., the number of surviving plants was higher than other treatments, but no virus free plants were obtained.

2- Only one plant was virus free, when cloves were exposed to hot air at 50, 55 and 60 °C for 50, 50, and 40 min., respectively.

3- The number of surviving plants was decreased by increasing the degree of temperature as well as the exposing time.

Data demonstrated in Table (6) revealed that only one virus free plant was found when cloves of cv. Balady were treated with hot air (55 °C) for 40 and 50 min. and with hot air (60 °C) for 30 min. Also results showed that the number of surviving plants was higher than other treatments when cloves were exposed to hot air (35-50 °C) but no virus free plants were obtained.

Heat therapy or combining *in vitro* with thermotherapy and meristem culture to produce virus free garlic plants were reported by several investigators (Lin 1985, Ding *et al.*, 1988 and Conci and Nome, 1991).

In the Assian Vegetable Research and Development Center (AVRDC 1994) reported that bulbs of each three garlic lines were subjected to heat treatment which consisted of submerging the cloves in tap water for 20 h and then placing them for 2 h in a water bath was set at 50 °C. After the treatment, meristems of 0.3 mm were excised and placed on MS medium and 10 days later transferred to MS medium amended with 50 Mg/L virazole for 6 weeks, then shoots of 0.1-0.2 cm were rexcised and placed on MS medium. ELISA tests showed that virus elimination was approximately 90%.

Table (4): **Effect of virazole *in vitro* on elimination of garlic viruses**

Cultivars	No. of excisting meristems	No. of surviving plantlets	No. of plantlets treated with virazole	No. of virus free plantlets	percentage of virus free plantlets
Chinese	43	20	20	20	100
Balady	106	20	20	20	100

Table (5): Effect of treatment with hot water on cloves of garlic cv. Chinese for garlic viruses elimination

Treatment with hot water		Cv. Chince			Cv. Balady		
Degree °C	Period min.	No. of survival plants	No. of infected plants	No. of virus free plants	No. of survival plants	No. of infected plants	No. of virus free plants
40	25	10	10	-	8	8	-
40	30	10	10	-	7	7	-
40	45	9	9	-	6	6	-
40	60	6	6	-	5	5	-
40	75	7	7	-	4	4	-
45	25	10	10	-	8	8	-
45	30	9	9	-	6	6	-
45	45	7	7	-	5	5	-
45	60	6	6	-	4	4	-
45	75	5	4	1	1	1	-
50	25	10	10	-	6	6	-
50	30	5	5	-	5	5	-
50	45	4	4	-	3	3	-
50	60	3	2	1	2	1	1
50	75	1	1	-	1	-	-
55	25	7	6	1	4	4	-
55	30	3	2	1	3	1	2
55	45	1	1	-	1	1	-
55	60	-	-	-	-	-	-
55	75	-	-	-	-	-	-
Control	-	9	9	-	6	6	-

Ten cultivated cloves were used for each degree and each period.

Table (6): Effect of treatment with hot air on cloves of garlic cv. Chinese and balady for garlic viruses elimination

Treatment with hot air		Cv. Chinese			Cv. Balady		
Degree °C	Period min.	No. of survival plants	No. of infected plants	No. of virus free plants	No. of survival plants	No. of infected plants	No. of virus free plants
35	30	10	10	0	8	8	0
35	40	10	10	0	8	8	0
35	50	8	8	0	7	7	0
35	60	2	2	0	2	2	0
35	75	0	0	0	0	0	0
35	90	0	0	0	0	0	0
40	30	9	9	0	8	8	0
40	40	9	9	0	7	7	0
40	50	8	8	0	6	6	0
40	60	2	2	0	2	2	0
40	75	0	0	0	0	0	0
40	90	0	0	0	0	0	0
45	30	9	9	0	8	8	0
45	40	8	6	0	6	6	0
45	50	7	2	0	3	3	0
45	60	1	1	0	0	0	0
45	75	0	0	0	0	0	0
45	90	0	0	0	0	0	0
50	30	9	9	0	7	7	0
50	40	6	6	0	5	4	1
50	50	3	3	0	3	3	0
50	60	1	1	0	0	0	0
50	75	0	0	0	0	0	0
50	90	0	0	0	0	0	0
55	30	5	4	1	4	3	1
55	40	4	2	0	3	2	1
55	50	3	0	0	2	1	1
55	60	0	0	0	0	0	0
55	75	0	0	0	0	0	0
55	90	0	0	0	0	0	0
60	30	4	3	1	3	2	1
60	40	1	0	1	1	1	0
60	50	0	0	0	0	0	0
60	60	0	0	0	0	0	0
60	75	0	0	0	0	0	0
60	90	0	0	0	0	0	0

Ten cultivated cloves were used for each degree and each period.

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