African Crop Science Journal, Vol. 24, No. 3, pp. 305 - 315
Printed in Uganda.
ISSN 1021-9730/2016 $4.00

DOI: http://dx.doi.org/10.4314/acsj.v24i3.7

IN VITRO TECHNIQUE FOR SELECTING ONION FOR WHITE ROT DISEASE-RESISTANCE

SAYED A.-M. OSMAN, KASEM Z. AHMED, ABDEL-RAHEM T. ABDEL-RAHEM and ABDEL-RAHEM A. MOHAMED[1]

Department of Genetics, Faculty of Agriculture, Minia University, El-Minia, Eg-61519, Egypt
[1]Field Corps Research Institute, Agriculture Research Centre, Giza, Egypt
Corresponding author: sayedosman12@yahoo.com

(Received 29 May, 2015; accepted 15 August, 2016)

ABSTRACT

In vitro selection is one of the most effective and efficient techniques for plant improvement. This is due to its ability to isolate plants with the desired character(s), either by applying a selection agent on the culture media to drive the selection of somaclones with the required character(s), or by establishing particular conditions that change in the genomes of somaclones toward the required character. The objective of this study was to identify a suitable protocol for *in vitro* selection of *Allium* white rot disease (*Sclerotium cepivorum*) tolerance in commercial Egyptian onion varieties, namely Giza 20, Giza 6 and Beheri Red. Oxalic acid (OA), the phytotoxin produced by *Sclerotium cepivorum*, was used as the selective agent. Seeds of the three Egyptian varieties were germinated on four concentrations (0.0, 0.02, 0.2, 2 and 20 mM) of Oxalic acid. Among the tested cultivars, Beheri Red had the highest germination frequency (52%) at all concentrations tested, followed by Giza 20 (42.6%), and Giza 6 at (32%). Cotyledon explants from the varieties were cultured on toxic MS_{BDK} medium, supplemented with 0, 3, 6 and 12 mM OA. The survival of calli on MS_{BDK} free toxic medium was 70.7% for all tested cultivars; however, MS_{BDK}-stressed medium, with 3 mM OA reduced the viable calli to 42.1%. The highest OA concentration (12 mM) completely inhibited calli induction from cotyledons explants. A medium supplement with 3 mM OA retarded 80% of calli growth. Among 156 tested calli of Beheri Red, only 23 calli (14.7%) survived on toxic medium for 45 days. Similarly, there was 15.6% survival for Giza 20 calli, while 40.1% of the Giza 6 calli survived. Plantlets were regenerated from surviving calli and transplanted onto *ex vitro,* and formed bulb after acclimatisation.

Key Words: *Allium cepa*, oxalic acid, *Sclerotium cepivorum*

RÉSUMÉ

La selection *In vitro* est l'une des techniques les plus efficaces en amelioration des plantes. Ceci est dû á la capacité qu'á cette technique de permettre l'isolation des plants avec des caractères désirés. Ceci se fait de deux manières; soit en appliquant un agent de selection sur le milieu de culture afin d'orienter la sélection somaclonale de façon á préserver le caractère desiré, ou, en créant des conditions particulières visant á modifier le genome afin dans le sens des caractères voulus. L'ojectif de l'étude était d'identifier un protocole adéquat pour la selection *in vitro* de la tolérance á la maladie de pourriture blanche (*Sclerotium cepivorum*) chez les variétés commerciales d'oignon en Egypte, en loccurence, Giza 20, Giza 6 et Beheri Red. L'acide oxalique (OA), la phytotoxine produite par *Sclerotium cepivorum*, ont été utilisés comme agent de sélection. Les semences des trois variétés Egyptienne d'oignon ont été cultivées sur quatre milieu de culture de différentes concentrations d'acide oxalique (0.0, 0.02, 0.2, 2 et 20 mM). parmi les cultivars testés, Beheri Red avait la fréquence de germination la plus (52%) sur tous

les quatre milieu de culture, puis vint Giza 20 (42,6%) et Giza 6 (32%). Des explants de cotyledons ont été cultivés sur milieu toxique MSBDK, additionné de 0, 3, 6 et 12 mM de OA. Les calls survivants sur milieu toxique mais ne contenant pas de MSBDK était de 70.7% pour tous les cultivars testés; néanmoins, le milieu contenant MSBDK, avec 3 mM de OA a causé la réduction des calls viables de 42.1%. la plus forte concentration de OA (12 mM) a causé une inhibiton complete de la régération des calls á partir des explants de cotyledons. Un milieu additionné de 3 mM de OA á retardé la croissance des calls de 80%. Parmi les 156 calls de Beheri Red testés, seuls 23 calls, soit 14,7%, ont survécu sur milieu toxique pendant 45 jours. De même, il y avait 15,6% de calls survivants Giza 20 calli, tandis que 40,1% des calls de Giza 6 ont survécu. Des plantules ont été régénéré á partir des calls survivants et ceux ci ont été transplanté sur milieu naturel, ils forment des bulbes d'oignons après un temps d'acclimatation.

Mots Clés: Allium cepa, acide oxalique, *Sclerotium cepivorum*

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important vegetable crops in Sub-Saharan Africa. Onion is valued as a spice because of its flavour, high nutritional value, medicinal properties and antioxidant additive for food (Dini *et al*., 2008). In 2011 the dry-land area cultivated with onion reached 51,474.00 hectares in Egypt, rising from 3,761,000 hectares, and producing 1,903,000.00 metric tonnes (FAO, 2013). Thus, onion is considered an important cash and export crop in Egypt. *Allium* white rot (AWR) is one of the most serious diseases of onion and other *Allium* spp.

Like many other crops, onion is susceptible to several plant pathogens including soil-born fungi; such as molds, fungi, bacteria and nematodes (Davis *et al*. 2007; Francisco *et al*., 2011). The causal agent of the disease is *Sclerotium cepivorum,* which is a fungal pathogen and usually produces sclerotia in the soil (Ulacio-Osorio *et al*., 2006). The sclerotia remain in the soil in the absence of host plants, for more than 20 years. They begin to germinate only in the presence of *Allium*-specific root exudates, thus making the disease highly specific to *Allium* species. The sclerotia penetrate their host plant causing AWR disease (Maude, 2006; Davis *et al*., 2007).

Germination of sclerotia is stimulated by volatile thiols and sulphides, which are released by microorganisms metabolising alk(en)yl cysteine sulphoxides, secreted from the roots of *Allium* spp. (Davis *et al*., 2007). The disease has been found in most of the regions where onions are cultivated (Stewart and McLean, 2007), and to date no system of control has been shown to fully prevent the occurrence of the disease in Egypt.

Sclerotium cepivorum damages Allium tissue during infection, by degrading plant cell walls ahead of hyphal elongation, through the secretion of a fungal toxin, oxalic acid (OA) (Maude, 2006). OA plays a central role in white rot pathogenicity (Maude, 2006). Some researchers suggest that oxalic acid produced by pathogens such as *S. sclerotiorum,* deregulates guard cells (Guimaraes and Stotz, 2004), either makes surface penetration easier, and allows the pathogen to more easily leave an infected leaf to produce sclerotia and propagate further infection. This stomatal deregulation may also be the cause of the characteristic of leaves infected with the generalist pathogen *S. sclerotiorum.*

The role of oxalate as an essential virulence factor for *S. sclerotiorum* was demonstrated by the observation that mutants deficient in oxalate biosynthesis are less pathogenic than wild type fungus (Godoy *et al*., 1990). Oxalate-deficient *S. sclerotiorum,* which are an important source of inoculum in the field and in *in vitro* cultivation is unable to produce oxalate during the infection of petals (Rejanel and Stotz, 2004; Chamandoosti, 2009).

Control of AWR has proved difficult because of the persistence of the sclerotia. Various chemical, biological, and physical measures have been examined for the control of the disease, including fungicides (Zewide *et al*., 2007a), soil amendments (Brewster, 2008), soil solarisation (Melero-Vara *et al*., 2000), composts (Smolinska, 2000; Coventry *et al*., 2005) and biological control agents (Clarkson *et al*., 2002; Coventry *et al*.,

2006; Zhang *et al*., 2013; Elsherbiny *et al*., 2015; Mahdizadehnaraghi *et al*., 2015). In addition, a number of studies have attempted to mimic the natural phenomenon of sclerotia germination with chemicals (Davis *et al*., 2007). While these methods have shown promise, problems still remain, including enhanced degradation of fungicides in the soil (Pung *et al*., 2007) and inconsistent control (Melero-Vara *et al*., 2000).

Plant improvement through somaclonal variation and *in vitro* selection are some of the best techniques for obtaining plant genotypes tolerant to biotic or abiotic stresses, such as drought, high salinity, acid soil and plant disease tolerance. Utilising somaclonal variation and *in vitro* selection techniques for obtaining potentially disease-resistance plants, has been demonstrated in a number of economically important crop plants (Jayasankar and Gray, 2003). Since the late 1970s, the process of *in vitro* selection has been applied to several cell culture systems, to generate mutants with useful agronomic traits, such as disease resistance (Jayasankar and Gray, 2003). *In vitro* selection was considered as a supplementary tool to the classical selection in breeding disease resistant cultivars, because of several advantages, such as faster testing of large numbers of individuals in a small space, easier manipulation of mutants, and the production of somaclones and haploids with higher genome variability (Ahmed *et al*., 1996).

The objective of this study was to establish a suitable tissue culture protocol for plant regeneration and *in vitro* selection of resistance to *Allium* white rot (*Sclerotium cepivorum*) in commercial Egyptian onion varieties.

MATERIALS AND METHODS

Plant material. Seeds of three local commercial onion (*Allium cepa* L.) cultivars, Giza 20, Giza 6, Beheri Red, were kindly provided by the Seds Agriculture Research Station, Agriculture Research Center (ARC), Ministry of Agriculture, Seds, Egypt. These cultivars are sensitive to *Allium* white rot (*Sclerotium cepivorum*) disease in onions (Davis *et al*. 2007). Plant regeneration was achieved using the following protocol: Seeds were surface sterilised in a 0.15% HgCl2 solution (w/v) for 2 min. It was followed by soaking for 2 min in 70% ethanol, then 15 min in 20% solution of commercial bleach (Clorox®) containing 5.25% sodium hypochlorite, and subsequently rinsed 4 times with sterile water. The sterile seeds were cultured in germination 1/2 MS medium (half strength of Murashige and Skoog (1962) salts), without plant growth regulators (PGRs) and supplemented with 0.8% (w/v) agar and 1.5% (w/v) sucrose. The pH of the medium was adjusted to 5.8 and autoclaved at 121 °C for 20 min. After two weeks, cotyledons of seedlings (4-6 mm) were removed for use as explants.

Calli induction. Explants were cultured on calli induction sterile MS medium, supplemented with 2 mg l^{-1} benzylaminopurine (BAP), 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg l^{-1} kinetin (Kin), 30 g l^{-1} sucrose and 8 g agar/liter at pH 5.8. Three explant pieces were cultured in each vial (250 ml baby food jars). The explants were incubated in vials at 25±2 °C, in the dark for 45 days.

In vitro selection of OA resistant plants

Effect of OA on onion seed germination. Seeds of the three cultivars were imbibed in distilled water containing various concentrations of OA, $C_2H_2O_4$ (0.0, 0.02, 0.2, 2 and 20 mM). Fifteen seeds for replication and three replicates for each concentration from each genotype, were germinated in Petri-dishes and cultured at 27 °C with a 16 hr light per 8 hr dark cycle. The percentage of germination was scored after 15 days.

Effect of OA on onion explants. Cotyledons from the three onion cultivars were cultured on toxic media, MS_{BDK} medium, supplemented with varying concentrations of OA of 0, 3, 6 and 12 mM. MS_{BDK} is MS containing 2 mg l^{-1} BAP, 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} Kin, 30 g l^{-1} sucrose, 8 g l^{-1} agar at pH 5.8. Explants were incubated at 25±2 °C in the dark for 45-day. The number of formed calli were scored 4-6 weeks after the initiation of the experiment, and presented as percentage of calli induction. Induced calli were transferred onto a

regeneration medium, and after 6 weeks, the percentage of regenerated plantlets per calli was recorded.

Effect of OA on onion calli

LD_{80} of OA as toxic. To determine a suitable selection protocol, Giza 20, which showed the highest tolerance compared to Giza 6 and Beheri Red was adopted for this segment of the study. Giza 20 calli were divided into small pieces (20-30 mg) and placed on different concentrations of toxic medium (MS_{BDK} medium supplement with 0, 3 ,6 and 12 mM OA). Four pieces of calli, cultured on each petri-dish (Ø 5 cm), served as a single replicate. Five replicates were established for each treatment. The calli were incubated at 25-27 °C in the dark for 4 weeks. Fresh weight and relative growth rates of calli growing on each of the four levels of phytotoxin, were determined at 4 weeks. LD_{80} was established for reference to approximately 80% retarded growth of calli.

***In vitro* selection procedures.** For the initial selection of white-rot tolerant plants, small pieces (2 mm) of six week-old embryonic calli from the three cultivars (Giza 20, Giza 6 and Beheri Red) were subjected to MS_{BDK} culture toxic medium (MS containing 2 mg l^{-1} BAP, 1 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} Kin, 30g l^{-1} sucrose, 8g 1 l^{-1} agar), supplemented with 3 mM OA (this concentration of OA was selected for *in vitro* selection) (Fig. 1B). For selection of white rot tolerance, six week-old calli, derived from the three onion cultivars, were used. Small pieces (approx. 20-30 mg) of embryogenic calli were exposed to MS_{BDK} culture toxic medium, supplemented with 3 mM oxalic acid (this concentration was chosen based on our above experiment result).

After 45 days on selective medium, putative tolerant/resistant calli were transferred to regeneration medium (non-toxic MS medium containing 0.2 mg l^{-1} NAA, 2 mg l^{-1} BAP, 60 g l^{-1} sucrose and 8g l^{-1} agar). The calli were incubated at 25-27°C under fluorescent light 1000 lux, (16 hr day^{-1}) for 5-6 weeks.

The pH of all media used in this study (which contain OA) were adjusted to 5.8, after adding the OA before autoclaving at 121°C for 20 min. The percentages of regenerated shoots or plantlets were calculated per calli after 6 weeks of culture. Regenerated plants from survivor calli were transplanted in plastic pots (Ø 5 cm) filed with peat moss-soil mixture (1:1, v/v) and adapted under greenhouse conditions.

Statistical analysis. Data were statistically analysed using a randomised complete block design (RCBD) in a factorial arrangement according to Sndecor and Cochran (1990). Specifically, the Statistical Package for the Social Sciences (SPSS) V.10 (1999) was used for analysis. For mean separation, the Least Significant Differences at $P<0.05$ was used.

RESULTS

Effect of OA on onion seed germination. There were strong genotypic and treatment differences with respect to germination mean and percentage of onion seeds of three Egyptian onion cultivars (Table 1). Among the tested cultivars, Beheri Red exhibited the highest germination frequency (52%) at all tested concentration, followed by Giza 20, and Giza 6.

Explant growth and callus initiation. There were significant differences between callus initiation rates for each of the different concentrations of OA; but no genotypic differences were observed for the three Egyptian onion commercial cultivars (Table 2). Calli production decreased with increasing OA concentration. Explants cultured on MS_{BDK} toxic free medium produced an overall average of 70.7% calli produced for all tested onion cultivars.

However, only 42.1% of explants grown on MS_{BDK} toxic medium with 3 mM OA produced calli compared to 29.4% for explants grown on 6 mM OA toxic medium (29.4%). Callus initiation was completely inhibited by the highest OA concentration (12 mM) (Fig. 1A). At 3 mM OA, 51.6% of the cotyledons of Giza 20 produced calli as the highest percentage, followed by Beheri Red (42.2%), and Giza 6 (33.3%) calli (Fig. 1B).

The effect of OA on the regeneration rate of the three onion cultivars "Giza 20, Giza 6 and Beheri Red" are shown in Table 3 and Figure 1C. The grand mean number of regenerated shoots per calli was 5.2 shoots per callus achieved on

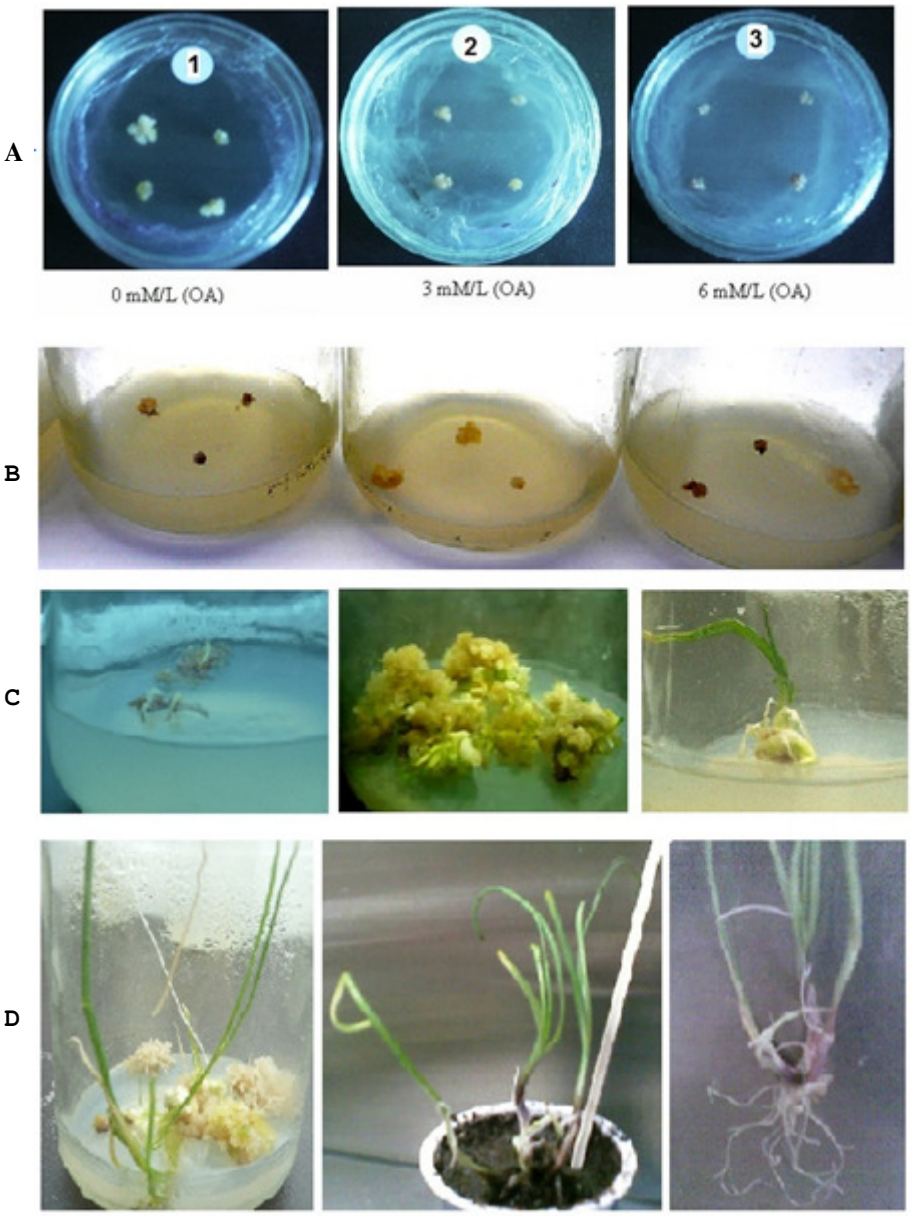

Figure 1. *In vitro* selection of onion plants resistant to oxalic acid (OA) in 3 commercial Egyptian cultivars (Giza 20, Giza 6 and Beheri Red). (A) Effect of MS_{BDK} toxic medium supplemented with different concentration of OA (0, 3 and 6 mM) on callus induction and growth. (B) Different response of onion calli growing on MS_{BDK} toxic medium supplemented with 3 mM OA. (C) Response of survivor onion calli on non-toxic MS regeneration medium. (D) From left to right, regenerated onion plantlets derived from calli grown on 3 mM OA containing MS medium, and transplanted putative resistant-plants in plastic pot for acclimatisation, and finally an intact plant with a formed bulb after acclimatisation.

TABLE 1. Germination mean and percentage of onion seeds of three Egyptian onion commercial cultivars "Giza 20, Giza 6 and Beheri Red" on four different oxalic acid (OA) concentrations (0, 0.02, 0.2, 2 and 20 mM)

Oxalic acid concentration	Cultivar							
	Giza 20		Giza 6		Beheri Red		Mean	
	Germination mean	Germination (%)	Germination mean	Germination (%)	Germination mean	Germination (%)	Germination mean	Germination (%)
Control	4.33±0.33	73.3±6.6	3.00±0.57	60±11.5	3.67±0.33	86.7±6.6	3.67±0.29	73.3±5.7
0.02 mM	4.33±0.33	73.3±6.6	2.67±0.33	53.3±6.6	3.67±0.33	86.7±6.6	3.56±0.33	71.1±5.8
0.2 mM	3.33±0.88	53.3±6.6	2.33±0.33	46.7±6.6	2.67±0.33	66.7±17	2.78±0.32	55.5±6.5
2 mM	1.00±0.00	13.3±6.6	0±0.0	0±0.0	0.67±0.33	20±0.0	0.56±0.18	11.1±3.5
20 mM	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
LSD (0.05)			Concentration =11.79%		Cultivar = 9.13%			

TABLE 2. Explants forming calli on toxic MS media at the different levels of oxalic acid (OA) for three Egyptian commercial cultivars

Oxalic acid concentration (mM/L)	Cultivar			Mean Calli
	Giza 20 Calli (% ± SE)	Giza 6 Calli (% ± SE)	Beheri Red Calli (% ± SE)	(% ± SE)
0	70.5±2.44	71.6±11.6	70±15.3	70.7±5.6
3	51.6±0.66	33.3±19.2	42.2±13.5	42.1±7.2
6	31.6±18.7	30±17.3	26.6±6.7	29.4±7.7
12	0±0.0	0±0.0	0±0.0	0±0.0
LSD (0.05)	Concentration=17.67		Cultivar = N.S*	

*non-significant diferences

TABLE 3. Effect of toxic oxalic acid (OA) on regeneration ability of surviving onion calli for commercial Egyptian cultivars on calli initiation medium

Oxalic acid concentration in calli initiation media	Cultivar			Mean
	Giza 20 Shoots calli^{-1}	Giza 6 Shoots calli^{-1}	Beheri Red Shoots calli^{-1}	
0 mM (control)	6.0±1.50	3.7±0.3	6.0±1.5	5.2±0.7
3 mM	1.3±0.33	2.4±0.88	2.7±1.2	2.1±0.5
6 mM	0±0.0	0±0.0	0±0.0	0±0.0
LSD (0.05)		Concentration=1.52	Cultivar = N.S*	

*non-significant diferences

calli growing on OA free medium across cultivars (Table 3). The presence of 3 mM OA in the medium decreased the mean number of shoots/calli from 5.2 to 2.1. Significant differences in the ability to generate shoots/calli were observed in genotypes Giza 20 and Beheri red, but not for Giza 6. The highest concentration completely inhibited the regeneration process (Fig. 1D).

Toxicity of OA on onion calli.

LD_{80} of *S. cepivorum* toxic OA. Fresh weight and relative growth rates of calli growing on the four different levels of phytotoxic were evaluated for Giza 20 cultivar (Table 4). There was a wide range of growth rates (fresh weight) for onion calli grown on toxic media, with different concentration of oxalic acid. Giza 20 calli growth on OA-free medium (control) increased 5.08 times from the starting materials (control); while calli grown on toxic medium supplemented with 3 mM OA increased only 1.05 times (Table 4). Lower growth was recorded for toxic medium with 6 mM and 12 mM (68.34 and 44.42%, respectively), representing 13.45% and 8.74% with respect to controls.

***In vitro* selection procedures.** Exposure of the calli to the toxic medium resulted in differing survival scores for each of the three cultivars (Table 5). The calli from Giza 6 had the highest survival percentage (Table 5). However, 100% of the Beheri Red calli regenerated in contrast with 70.7 and 26.6% for Giza 6 and Giza 20,

TABLE 4. Percentage of fresh weight of Giza 20 onion calli grown on MS_{BDK} toxic medium supplemented with different concentration of oxalic acid

OA concentration	Calli weight (mg)*	Calli after 30 days on toxic medium (mg)*	Average increase in weight (mg)	Weight increase (%)±SE	Weight increase respect to control (%)
0 mM (control)	21.8	132.6	110.8	508.26±137	100
3 mM	51.7	106.49	54.79	105.97±31	20.85
6 mM	39.8	67	27.2	68.34±11	13.45
12 mM	43.9	63.4	19.5	44.42±13	8.74
LSD (0.05)				209	

*Mean of fresh weight of 24 calli each

TABLE 5. *In vitro* selection of onion calli resistant to 3 mM oxalic acid (OA) and regeneration frequency of surviving calli after selection period of Egyptian commercial cultivars

Cultivar	*In vitro* selection			Regenerating calli			
	Number of Calli cultured on toxic medium	Number of surviving calli	Percentage of surviving calli	Calli No.	Calli %	Number of shoots	Shoots/calli
Giza 20	96	15	15.6±3.9	4	26.6	5	1.25±0.75
Giza 6	102	41	40.1±6.1	29	70.7	14	0.48±0.27
Beheri red	156	23	14.7±2.4	23	100	32	1.39±0.61
LSD (0.05)			11.8				N.S*

*non-significant diferences

respectively. Beheri Red also had the highest number of regenerated shoots per callus, compared to 1.25 for Giza 20 and 0.48 for Giza 6. Several shoots were rooted to develop healthy plantlets, which were transplanted to plastic pots and adapted under greenhouse condition where the intact plants were able to form bulbs (Fig. 1D).

DISCUSSION

Onion exhibited a wide range of sensitivity within the tested cultivars, and for seed germination on different OA concentrations, and Beheri Red exhibit the most tolerance. A higher OA concentration (20 mM) completely inhibited germination of all onion cultivars. Our results are in agreement with Chamandoosti (2009) who found that 20 mM of OA had a lethal effect on canola and all the explants died, and that no callusing, rooting and shooting occurred.

Callus induction was significantly affected by OA, and the extent of the response was OA concentration dependent (Table 2). However, the callus induction response it was the same for each cultivar and was independent of OA concentration. Moreover, the maintenance of callus regeneration was strongly affected by the presence of OA in culture medium (Table 3), which also decreased the mean number of shoots per callus. For example, the presence of 3 mM OA decreased the number of shoots per callus to 2.1 (Table 3).

Results of our research demonstrated that at 6 mM of OA concentration all explants died and no callusing, rooting or shooting were observed. Kumar *et al.* (2008) described a system for efficient plant regeneration *via* organogenesis and somatic embryogenesis of safflower (*Carthamus tinctorius* L.) cv. NARI-6 in fungal culture filtrates (FCF)-treated cultures. FCF was prepared by culturing *Alternaria carthami* fungal mycelia in selection medium for host-specific toxin production. They produced nine plantlets from organogenesis, and 24 plantlets from somatic embryogenesis were selected as FCF-tolerant plants. *Alternaria carthami* fungal spores sprayed on the leaves of FCF-tolerant plants showed enhanced survival rate over control plants, which plants were more susceptible to fungal attack.

From our results, OA can be used for *in vitro* selection of onion plant resistant to OA. It is that medium supplemented with 3 mM OA retardes 80% of callus growth. Thus this OA concentration is suitable for *in vitro* selection in onion as demonstrated by our study.

Recent advances in molecular characterisation of stress-related responses and the emergence of sensitive molecular analytical tools, have reinvigorated work on *in vitro* selection. This technology is easy to use and not encumbered by intellectual property issue and social concerns that currently inhibiting development of transgenic crops. Thus, it is an attractive complement to existing crop improvement strategies (Jayasnkar and Gray, 2003).

American chestnut wild type callus tissue and calli transformed with an oxalate oxidase gene were cultured on media containing various concentrations of oxalic acid. Thermogravimetric analysis (TGA) was employed to test the cellulose, hemicellulose and lignin content of the calli. In the presence of oxalic acid, wild type tissues demonstrated a significant decrease in lignin content and an increase in cellulose content, while transformed tissues did not. Transforming American chestnut with an oxalate oxidase gene may prove to be useful in preventing changes in the cell wall composition caused by the oxalate produced during *C. parasitica* infection and could possibly enhance resistance (Welcha *et al*., 2007).

In vitro selection has been used not only in response to pathogen infection but also in many cases in response to abiotic stresses. Mercado *et al*. (2000) used of growth of apical stem sections and adventitious organogenesis to evaluate salinity tolerance in cultivated tomato (*Lycopersicon esculentum* L.) and resulted that this approach may not be a reliable tool to evaluate salt tolerance in tomato.

Oxalic acid produced by the fungal pathogen *Sclerotinia sclerotiorum* has a primary role in the pathogenicity of this white-rot disease in onion. Mutant strains of this fungus, deficient in oxalate productions are avirulent. Furthermore,

transgenic soybean (*Glycine max* L. Merr) expressing oxylate oxidase that catalyses the breakdown of oxalic acid, reduces the growth of the pathogen in indoor seedling studies. Cober *et al*., (2003) demonstrating that susceptibility of onion in *in vitro* conditions is likely to be similar to susceptibility for whole plants under *in vivo* conditions.

Oxalic acid has been used for *in vitro* selection studies in many important crops affected by *Sclerotium* fungi (Chamandoosti *et al*., 2006; Chamandoosti, 2007; Chamandoosti, 2009). Chamandoosti (2009) reported that the addition of 0.20 mM OA was needed for *in vitro* canola selection media in order to produce canola plants resistant to *Sclerotinia sclerotiomm*. Chamandoosti (2009) reported that the mean diameter of calli and mean number of regenerated roots and shoots of hypocotyl explants of 7 days old canola seedling growing on 0.20 mM OA was higher than in OA-free medium. It is possible that in these experiments the gradual adaptation of hypocotyl explants in the toxic callusing culture medium and subsequent transfer of obtained calli to organogenesis medium generated tolerant or resistant whole plants.

By using *in vitro* selection protocol, we could select calli resistant to OA and from these calli the complete plants were regenerated. Healthy plants were transplanted and grown under greenhouse condition and many of them formed bulbs. These bulbs were subjected to field challenges caused by *Allium* white rot(*Sclerotium cepivorum*). However, utilising *in vitro* selection techniques for obtaining potentially disease-resistance plants has been demonstrated in a number of economically important crop plants (Jayasankar and Gray, 2003; Savita *et al*., 2011; Zhang *et al*., 2012; Ravaei *et al*., 2015). In conclusion, results of this research clearly demonstrate that the gradual incremental addition of oxalic acid concentration to onion calli media acts as a selection pressure for the production of tolerant onion lines and that 3 mM OA is the optimum concentration for the initiation (gradually adaptation) of the production protocol. This may indicate that Beheri Red seeds were more OA-tolerant than the other two Egyptian cultivars.

ACKNOWLEDGEMENT

We are grateful to Prof. Dr. Melvin J. Oliver, (Research Leader, Plant Genetics Research Group, USDA-ARS-MWA, University of Missouri, Curtis Hall, Columbia, MO , USA) for assistance with preparation of this manuscript.

REFERENCES

Ahmed, K.Z., Mesterhazy, A., Bartok, T. and Sági, F. 1996. *In vitro* techniques for selecting wheat (*Triticum aestivum* L.) for Fusarium-resistance. II. Culture filtrate technique and inheritance of Fusarium-resistance in the somaclones. *Euphytica* 91: 341-349.

Brewster, J.L. 2008. Onions and other vegetable Alliums. 2nd Ed. Crop Production Science in Horticulture Series, Volume 15.

Clarkson, J.P., Payne, T., Mead, A. and Whipps, J.M. 2002. Selection of fungal biological control agents of *Sclerotium cepivorum* for control of white rot by sclerotial degradation in a UK soil. *Plant Pathology* 51: 735-745.

Chamandoosti, F. 2007. Effect of sodium chloride on establishment of calli and organogenesis in *Brassica napus* L. *Pakistan Journal of Biological Sciences* 10: 3880-3884.

Chamandoosti, F. 2009. *In vitro* selection: A novel source of resistance to *Sclerotinia* stem rot in Canola (*Brassica napus* L.). *Asian Journal of Plant Physiology* 3: 8-13.

Chamandoosti, F., Majd, A. and Mehrabian, S. 2006. *In vitro* plant regeneration from calli of cotyledons in canola (*Brassica napus* L.). *Pakistan Journal of Biological Sciences* 9: 302-306.

Cober, E.R., Riouxb, S., Rajcan, I., Donaldson, P.A., and Simmonds, D.H. 2003. Partial resistance to white mold in a transgenic soybean line. *Crop Science* 43: 92-95.

Coventry, E., Noble, R., Mead, A. and Whipps, J.M. 2005. Suppression of *Allium* white rot (*Sclerotium cepivorum*) in different soils using vegetable wastes. *European Journal of Plant Pathology* 111: 101-112.

Coventry, E., Noble, R., Mead, A., Marin, F.R., Perez, J.A. and Whipps, J.M. 2006. *Allium* white rot suppression with composts and

Trichoderma viride in relation to sclerotia viability. *Phytopathology* 96: 1009-1020.

Davis, R.M., Hao, J.J., Romberg, M.K., Nunez, J.J. and Smith, R.F. 2007. Efficacy of germination stimulants of *Sclerotia* of *Sclerotium cepivorum* for management of white rot of garlic. *Plant Disease* 91:204-208.

Dini, I., Tenore, G.C. and Dini, A. 2008. Chemical composition, nutritional value and antioxidant properties of *Allium cepa* L. Var. *Troperana* (red onion) seeds. *Food Chemistry* 107: 613-621.

Elsherbiny, E.A., Saad, A.S., Zaghloul, M.G. and El-Sheshtawi, M.A. 2015. Efficiency assessment of the antifungal metabolites from Sclerotium cepivorum against onion white rot disease. *European Journal of Plant Pathology* 142: 843-854.

FAO. 2013. FAOSTAT: FAO Statistical Database. Food and Agriculture Organization, UN, New York, http://apps.fao.org=default.htm

Francisco, D.H., Angelica, M.P., Gabriel, M., Melchor, C.S., Raul, R., Cristobal, N. and Francisco, C.R. 2011. *In vitro* antagonist action of *Trichoderma* strains against *Sclerotium sclerotiorum* and *Sclerotium cepivorum*. *American Journal of Agriculture Biology Science* 6: 410-417.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclero tiorum* on *phaseolus vulgaris*. *Physiological and Molecular Plant Pathology* 37: 179-191.

Guimarães, R.L. and Stotz, H.U. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiology Preview*. pp. 1-9.

Jayasankar, S. and Gray, D.J. 2003. *In vitro* selection for diseases resistance in plants-an alternative to genetic engineering. *AgBiotechNet* 5: 1-5.

Kumar, J.V., Kumari, B.D., Sajatha, G. and Castaño, E. 2008. Production of plant resistant to *Alternaria carthami* via organogenesis and somatic embryogenesis of safflower cv. NARI-6 treated with fungal culture filtrates. *Plant Cell, Tissue and Organ Culture* 93: 85-96.

Mahdizadehnaraghi, R., Heydari, A., Zamanizadeh, H. R., Rezaee, S. and Nikan J. 2015. Biological control of garlic (*Allium*) white rot disease using antagonistic fungi-based bioformulations. *Journal of Plant Protection Research* 55:

Maude, R.B. 2006. Onion diseases. In: Cooke, M., Jones, D.G. and Kaye, B. (Eds.). The epidemiology of plant diseases, second Edition. Springer. pp. 506-509.

Melero-Vara, J.M., Prados-Ligero, A.M. and Basallote-Ureba, M.J. 2000. Comparison of physical, chemical and biological methods of controlling garlic white rot. *European Journal of P lant Pathology* 106: 581–588.

Mercado, J.A., Sancho-Carrascosa, M.A., Jimenez-Bermudez, S., Peran-Quesada, R., Pliego-Alfaro, F. and Quessada, M.A. 2000. Assessment of *in vitro* growth of apical stem section and adventitious organogenesis to evaluate salinity tolerance in cultivared tomato. *Plant Cell, Tissue and Organ Culture* 62: 101-106.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Pung, H., Cross, S., Florissen, P. and MacLeod, I. 2007. Investigating and developing fungicide options for onion white rot control in Australia. Peracto Pty Ltd. Publications.

Ravaei, A., Jonoubi, P. and Salmanian, A.H. 2015. Optimization of *in vitro* shoot regeneration and Agrobacterium-mediated transformation of *Brassica napus*. *International Journal of Biosciences* 6: 319-329.

Rejanel, G. and Stotz, H. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiology* 136: 3703-3711.

Savita, Gurdeep, S. V. and Avinash, N. 2011. *In vitro* selection of calli of *Citrus jambhiri* Lush. for tolerance to culture filtrate of *Phytophthora parasitica* and their regeneration. *Physiology and Molecular Biology of Plants* 17: 41-47.

Smolinska, U. 2000. Survival of *Sclerotium cepivorum* sclerotia and *Fusarium oxysporum* chlamydospores in soil amended with cruciferous residues. *Journal of Phytopathology* 148: 343-349.

Stewart, A. and McLean, K.L. 2007. Biological control of onion white rot. Chapter 6 in Biological control of plant diseases, 2007, The Haworth Press, Inc. New York, USA. pp. 123-148.

Ulacio-Osorio D., Zavaleta-Mejía, E., Martínez-Garza, A. and Pedroza-Sandoval, A. 2006. Strategies for management of *Sclerotium cepivorum* Berk. in garlic. *Journal of Plant Pathology* 88: 253–258.

Walker, A., Brown, P.A. and Entwistle, A.R. 1986 Enhanced degradation of iprodione and vinclozolin in soil. *Pesticide Science* 17: 183-193.

Welcha, A., Stipanovicb, A.J., Maynardc, C.A. and Powella, W.A. 2007. The effects of oxalic acid on transgenic Castanea dentate calli tissue expressing oxalate oxidase. *Plant Science* 172: 488-496.

Zewide, T., Fininsa, C. and Sakhuja, P. K. 2007. Management of white rot (*Sclerotium cepivorum*) of garlic using fungicides in Ethiopia. *Crop Protection* 26: 856-866.

Zhang, L.Q., Cheng, Z.H., Khan, M.A. and Zhou, Y. L. 2012. *In vitro* selection of resistant mutant garlic lines by using crude pathogen culture filtrate of *Sclerotium cepivorum*. *Australasian Plant Pathology* 41: 211-217.

Zhang, G., Wanga, F., Qin, J., Wanga, D., Zhang, J., Zhang, Y., Zhang, S. and Pan, H. 2013. Efficacy assessment of antifungal metabolites from *Chaetomium globosum* No. 05, a new biocontrol agent, against *Setosphaeria turcica*. *Biological Control* 64:90-98.