PRODUCTION OF DEVELOPED GRAPE ROOTSTOCKS USING *IN VITRO* MUTATIONS

By

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B.Sc. Agric. Sci. (Horticulture), Fac. Agric., Ain Shams Univ., ٢٠٠٠
M.Sc. Agric. Sci. (Pomology), Fac. Agric., Ain Shams Univ., ٢٠٠٧

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APPROVAL SHEET

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ABSTRACT

The present study was carried out during the period from ٢٠٠٨ to ٢٠١١. It aimed to induce and propagate an *in vitro* drought resistant mutagens of two Grape rootstocks *Vitis* spp., SO٤ and Freedom *via* the following steps:

First: Sterilizing shoot tips with Mercuric chloride ($HgCl_2$) and Sodium hypochlorite (NaOCl) at different concentrations and periods. This part results show that using ١٥ or ١٠% of Clorox for ١٥ minutes is beneficial in maintaining an aseptic culture since they record high survival and minor contamination percentages on the opposite of the higher sterilization concentrations and periods which are growth inhibitors. $HgCl_2$ treatments were lethal or inhibitors.

Second: a. Irradiating *in vitro* cultured plantlets of both rootstocks with Gamma rays at various doses to induce a mutation. Results indicate that all doses affected plantlets characters and ٤٠ Gy of Gamma rays is lethal for both rootstocks.

b. Testing irradiated plantlets susceptibility to drought through subculture on PEG ٦٠٠٠ containing medium at several concentrations. Five and ١٠ Gy irradiated plantlets had high survival and growth on high PEG concentrations except for the ١٠g/ L PEG which had dehydrated the plantlets within a week. Overall, the ٥ Gy dose seem to be a promoter for survival %, leaf area, No. of leaves and roots/ plantlet, and root length. The ١٠ Gy shared or succeeded the ٥ Gy dose in survival and most growth parameters, especially when combined with a low PEG concentration or under dehydration conditions. Twenty Gy of Gamma stimulated roots formation. However, the ٣٠ Gy dose inhibited survival and all growth parameters when cultured on different media. PEG strongly affected plantlets, since ٠,٥ g/ L enhanced survival and growth, on the contrary of the concentration of ٨ g/ L.

Third: RAPD- PCR technique proved a genetic structure alteration in Gamma irradiated plantlets. Polymorphism in Freedom reached about ٥٧,١ % with the primer OP-C٠٩, while the Primers OP-C١٣ and OP-D١٣ showed ٣٧.٥% polymorphism in SO٤ rootstock.

Both rootstocks biologically had the same trend under all treatments even if SO٤ had superior root length, while Freedom scored a greater number of roots. Freedom was more polymorphic with Gamma irradiation than SO٤.

Key words: Gamma irradiation, Mutation, Drought, PEG, RAPD- PCR, Grape

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CONTENTS

INTRODUCTION

Grape (*Vitis vinifera* L.) is one of the most favored fruits that have high nutritional benefits also it has a valuable commercial importance Worldwide; it is ranking the second fruit tree crop in Egyptian exportation after Citrus. This is why Grape takes a great interest from growers, which led to progressive stages in the World production and exportation. Grape production of Egypt reached about ١.٣٦٠.٢٥٠ tons cultivated in ١٥٣,٦٨٢ Feddan according to the statistics of FAO (٢٠١٠).

The Egyptian Agricultural strategy aims to continue expansion of fruit trees cultivation in the newly reclaimed areas and maximizing fruits exports.

Economic viability of a fruit tree production is linked directly to orchard productivity and management efficiency. Optimal levels of productivity require tree survival, managed vigor, and good marketable yields over the expected life span of the orchard.

The grower's choice of a rootstock is a quite important limiting factor when fruits are grown on dry, lime soils having pathogens, or other soil problems. In the Egyptian reclaimed regions, where one or more of these conditions is present, rootstocks that better address these site and vigor problems will become a crucial need. New rootstocks introduced through commercial nurseries need extensive testing to avoid commercial failures from rootstocks that prove poorly adaptation to local climatic and soil conditions. In addition, putative resistance of new rootstocks to soil problems has sometimes failed when planted in other production regions away from their origin.

Environmental stresses result in water deficiency for the plants, thus impairing its numerous biological roles. *In vitro* screening for stress tolerance will have its significance in identifying cultivars with optimal stress tolerance and productivity.

Most of rootstocks are hybrid Grape varities that are the product of a crossing of two or more *Vitis* spp. or an inter-specific crossings. Due to their excellent tolerance to powdery mildew, other fungal diseases, nematodes and phylloxera. Hybrid varieties exhibit a mixture of traits from their European, Asiatic, and North American parentage.

Two of the best and common Grape rootstocks in Egyptian vineyards are SO٤ or Teleki (*V. berlandieri* x *V. riparia* Michx.) and Freedom ((*V. champinii* Planch. x (*V. solonis* hort. Berol. ex Planch. x *V. othello*)). *Vitis berlandieri* is primarily known for good tolerance against soils with a high content of lime. It is poorly adapted to grafting. Various rootstocks resistant against both phylloxera and lime, and suitable for viticulture, were produced by crossing *Vitis berlandieri* and *Vitis riparia*, *Vitis rupestris* or *Vitis vinifera.*

SO٤ and Freedom characteristics shown in Table (١), however, their behaviour against drought conditions is not satisfactory which is why this study attempted to improve drought tolerance of these two rootstocks through genetic improvement *via* tissue culture and gamma irradiation inducing mutations techniques.

The high percentage of Gamma-ray irradiated mutants indicates that mutation breeding *via* Gamma-ray irradiation is an effective and highly successful approach for the generation of

commercial cultivars and new types for growers (Nakagawa, ٢٠٠٩).

In a mutant micropropagation program, it is of paramount importance to preserve the new genetic forms, but perhaps even more important is their genetic stability. Since the traditional methods of identifying Grape cultivars have relied on morphological characters whose expression is affected by developmental and environmental factors, RAPD this PCR- based technique has been adopted as a convenient and powerful means of detecting genetic differences among closely related forms (Gupta and Varshney ١٩٩٩ and Kim *et al.* ٢٠٠٢).

Table ١. Characteristics of SO٤ and Freedom Grape rootstocks.

Type	Vigor	Root system	Nematode resistance	Drought resistance	Alkalinity tolerance
SO٤	Moderate	Shallow	Moderate-High	Low	Fair
Freedom	Moderate-High	Moderate	High	Moderate-High	?

Source: Creasy and Creasy (٢٠٠٩).

Therefore, the present work was designed to study the following:

١. Effect of surface sterilization methods on aseptic cultures establishment of SO٤ and Freedom Grape rootstocks.

٢. Gamma irradiation doses capability in inducing drought tolerance mutation in both rootstocks and testing their mutants on PEG containing media.

٣. Induced mutations detection *via* RAPD- PCR analysis.

REVIEW OF LITERATURE

The previous work dealing with the aseptic cultures establishment, effect of Gamma irradiation and PEG in nutrient medium and examining mutagenesis using RAPD on different plants will be discussed as follows:

١. Sterilization

Sterilization of different *in vitro* cultured explants is a primary and basic step to establish an aseptic culture; so that several sterilization protocols were set up by researchers and the following are some of them:

On Artichoke (*Cynara scolymus*), Alphonse *et al.* (٢٠٠٢) revealed that the most effective sterilizing and disinfectant treatment for survival and bacterial contamination prevention was dipping shoot tips in ethanol ٧٠٪ (٣٠ sec) + sodium hypochlorite (NaOCl) ١,٥٪ (٢٠ min) + mixture of streptomycin and gentamycin each at ٥٠ mg/ L (٣٠ min).

While, Ebrahim *et al.* (٢٠٠٢) used sodium hypochlorite ٣٪ solution (contained ٠,١٪ Tween ٢٠) alone for ٢٠ minutes for surface sterilization of four Banana cultivars shoot tips (*Musa* spp.).

Also, Lee *et al.* (٢٠٠٢) mentioned that (١٠ mm) nodal stem cuttings of two Sweet Potato cultivars (*Ipomoea batatas* L.) were sterilized prior to culture with sodium hypochlorite (٥٪) for ١٠ minutes then washed with sterile distilled water.

Moreover, Ashok *et al.* (٢٠٠٣) sterilized two *Cucumis sativus* L. Cvs. flower buds with ٥٪ sodium hypochlorite solution for ٢٠ min,

then in a horizontal laminar air flow using ٠,١٪ mercuric chloride for ٥ min. Flower buds were rinsed several times in sterile distilled water.

Working on Apple (*Malus domestica*), El-Sabrout (٢٠٠٣) sterilized shoot tips (٢ mm) by immersing in ٧٠٪ ethanol for a minute then by dipping them in ٠,٢٪ solution of mercuric chloride ($HgCl_2$) for ٣ minutes, followed by three rinses in sterile distilled water.

Stevia rebaudiana seeds were sterilized by washing with ٠,١٪ detergent solution for ٥min, rinsed before surface sterilization in ٧٠٪ ethanol for ٣٠ sec followed by soaking in ١٪ commercial bleach (sodium hypochlorite) for a minute and rinsed thoroughly with sterile distilled water; Maharik and El- Gengaihi (٢٠٠٣).

Moreover, El-Hammady *et al.* (٢٠٠٥) indicated that dipping explants in ١٠٪ of sodium hypochlorite for ٨ minutes was effective in sterilizing Almond shoots (*Prunus dulcis* Mill).

In another study made by Mkuya *et al.* (٢٠٠٥) they indicated that Rice anthers (*Oryza sativa*) were surface disinfected by immersing in ٧٥٪ ethanol, then in ١,٠٪ antiformine for ٢٠ min and in ٣٪ hydrogen peroxide (H_2O_2) for ١٠ min, and finally they were washed ٣-٤ times with sterile distilled water.

Whereas, Sim (٢٠٠٦) surface sterilized Grape tissue (*Vitis vinifera*) by submersion in sodium hypochlorite (١٠٪) plus surfactant drops for ١٠ minutes. Tissue removed under aseptic conditions and serially transferred through three rinse containers containing sterile distilled water.

Also, Congfen *et al.* (٢٠٠٧) pointed out that the use of ٢٠,٠٪ sodium hypochlorite (٢٥ min) for Aloe (*Aloe barbadensis* Miller)

sterilization was less detrimental to the explants than ٠,١٪ mercuric chloride (١٠ min).

On the other hand, Yogeshwar *et al.* (٢٠٠٨) treated nodal segments explants (٢,٠ – ٣,٠ cm length) of *Bambusa tulda* with a quick dip for ٢٠ sec in ٧٠٪ ethanol before being administered three doses, *viz*., ٠,٠٥, ٠,١ and ٠,٢٪ each of aqueous sodium hypochlorite or mercuric chloride solution separately for ١٠ min. They showed that administration for ١٠ min of ٠,٠٥ and ٠,١٪ mercuric chloride to explants collected facilitated optimum culture establishment. ٠,١– ٠,٢٪ mercuric chloride enhanced aseptic culture establishment but inhibited bud break due to toxicity to explants.

Whereas, Sujatha and Kumari (٢٠٠٨) maintained aseptic culture of *Artemisia vulgaris* by surface sterilization of young nodal explants (١–١,٥ cm) with ٠,١٪ (w/v) aqueous mercuric chloride for ١– ٦ minutes.

El-Shamy *et al.* (٢٠٠٩) indicated that the highest percentage of survival and free contamination explants of *Pyracantha fortuneana* Roem were observed when shootlet explants soaked for ١ minute in ٧٠٪ ethanol, then immersed for ٢٥ minutes in ٤٠٪ Clorox plus drops of Tween ٢٠ and rinsed three times with sterile distilled water.

Hamouda *et al.* (٢٠٠٩) had surface sterilized *Pelargonium graveolens* L'Herit explants (١cm length; internodes segments) with ٠,٨٪ sodium hypochlorite for ٢٠ minutes.

On the other hand, Sakr *et al.* (٢٠١٠) compared two sterilizer's effects; sodium hypochlorite and hydrogen peroxide on *Echinacea purpura* seeds germination. They reported that the highest seed

germination percentage was obtained when coated seeds were sterilized with hydrogen peroxide.

Nodal segments (١,٠–١,٥ cm) of *Solanum nigrum* L. plants obtained from the field were sterilized as the following method: washed in tap water for ٢٠–٣٠ minutes then surface sterilized in laminar air hood with ٧٠٪ ethanol for ٣٠ s followed by ٠,٠٥٪ mercuric chloride for ٣–٤ minutes, and rinsed ٤–٥ times with sterile double distilled water, Satish *et al.* (٢٠١٠).

Wang *et al.* (٢٠١١) employed autoclaving and mercuric chloride sterilization for two-liquid-phase (TLP) soil slurry system and reported that no microorganisms were detected in the $HgCl_2$-sterilized soil slurries during the whole incubation period, indicating that the sterilization efficiency and effectiveness of $HgCl_2$ on soil slurry was much higher than those of autoclaving at ١٢١ °C for ٤٥ minutes.

٢. Gamma irradiation and Polyethylene Glycol

a. Gamma irradiation

Gamma is an electromagnetical ionizing type of radiation affects the molecules and ions in the cells of the living creatures; this is why it is useful in inducing mutations as the following reviews:

Pandini *et al.* (١٩٩٧) had induced mutations by Gamma radiation (٠, ٥, ١٠, ٢٠ and ٤٠ kR doses) for plant height in two Triticale cultivars (*X triticosecale* Wittmack). All radiation doses showed increase in genetic amplitude for this trait, being suitable for increasing variability in breeding programs.

Moreover, Yoshioka *et al.* (١٩٩٩) had maintained nine resistant mutants of Japanese Pear (*Pyrus communis*) to Black Spot Disease

derived by chronically irradiation of susceptible cultivars with Gamma-rays induced from acutely irradiated dormant scions. Although, irradiation had displayed unfavorable characteristics, all of the mutants showed an intermediate resistance to Black Spot Disease and conferred various levels of resistance.

Another study conducted by Lamseejan *et al.* (٢٠٠٠) on Gamma rays at ٠, ١٠, ٣٠, ٥٠, ٧٠, ٩٠ and ١١٠ Gy effect on *in vitro* culture of Chrysanthemum (*Chrysanthemum morifolium*). They noticed that, M١V٤ shoots irradiated at ٥٠Gy and over died. Lethal Dose ٥٠ (LD٥٠) was ١٤ Gy. Controls and treated plants at ١٠ Gy were able to survive and gave rise to the full grown plants. After ٦٠ days, control and treated plants were found to be different in average height and number of leaves. The treated plants had much more variation than the controls.

In this respect, Morishita *et al.* (٢٠٠١) concluded that the rate of mutation in Buckwheat (*Triticum aestivum* L.) M٢ generation was increased with Gamma ray dose. Gamma ray LD٥٠ estimated about ٣٥٠ Gy and ٦٠٠ Gy, respectively. However ٣٠٠-٤٠٠ Gy irradiation, lower than LD٥٠, was desirable to obtain higher mutation rates and fewer dead plants in this dose range.

Also, Lee *et al.* (٢٠٠٢) induced somaclonal variations in Sweet Potato (*Ipomoea batatas* L.) regenerates from Gamma irradiated embryogenic callus at different levels (٠, ٣٠, ٥٠, ٧٠ and ٩٠Gy). The frequency of morphological variants derived from the irradiated callus ranged from ٣- ٧,٨ % compared to ٠,١- ١,١٪ of that derived from the non- irradiated callus. Polymorphisms of the irradiated callus were ٦,١