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ESTABLISHMENT OF IN VITRO CULTURE OF GRAPES

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ABSTRACT:- The establishment of in vitro culture from shoot tip explants (meristemetic tissue) of grapes was investigated through tissue culture technique. These explants were collected from gene bank of Institute of Agricultural Biotechnology and Genetic Resources (IABGR), National Agricultural Research Centre (NARC), Islamabad, Pakistan. Fifteen accessions of grapes were surface sterilized and tested on 75% MS media for germination and initial growth parameters. Accession No. 020017 (Dakh-1) exhibited highest viability (100%), shoot length (4.12 cm) and nodes plant^{-1} (3.8). Moreover, it was found that response of cultures to different treatments was dependent both on accession and treatment duration. In conclusion, this protocol proved to be useful in optimizing the dose and duration of the treatment of grape explants with the surface disinfectant.

Key Words: Grapes; In vitro Establishment; Germplasm; In vitro Conservation; Tissue Culture; MS Media; Germination; Growth Parameters; Pakistan.

INTRODUCTION

In Pakistan, mainly in Balochistan province, grape is important being one of the cash crops. In 2009-10, total area under grapes was 15300 ha with total production of 64700 t and national yield of 4.2 t ha^{-1} (GoP, 2010).

The problem of high mortality and low rooting in the vegetative propagation exists in grapes and hence, it needs, use of in vitro techniques for conservation. Therefore, different grape accessions have been introduced from USA and Japan as a part of germplasm conservation and collection strategy for their utilization in the country (Sajid et al., 2003). Their sexual propagation is not feasible because resulting crop is not true to type. In vitro conservation is a tool of choice for the conservation of species which do not produce seeds and are propagated vegetatively or their seed cannot be used for production due to heterogeneity. In vitro conservation is used for storage of grapes germplasm in many ways like low temperature storage or retardation of growth with the help of osmotic compounds, for short and medium term conservation (Shuji et al.,1992).

Plant tissue culture is a novel technique and serves as a rapid mean of micro propagation of economically important and vegetatively propa-

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gated crops like grapes. Due to recent advances in techniques and application of plant cell culture and molecular biology, most of the current strategies for the application of biotechnology to crop improvement envisage the regeneration of thousand of plants from a single cell. However, each cell of such a crop is totipotent which contains necessary genetic information to produce true to type plants (Sagawa, 1984; Sagawa and Kunisaki, 1982).

The vegetative propagation involves mitotic cell division that duplicate the genotype of the plants and such crops whose seeds cannot be used for the production of true progeny lose their growth, yield and quality and 2-5 years old vines show low vigor (Scheck et al., 1998b). To overcome these problems, in vitro conservation is utilized for those plants which are vegetatively propagated. Now tissue culture laboratories are commercially producing a large number of herbaceous and horticultural species and several woody plants (Purohit, 2003). It is also vital for maintaining the genetic diversity of the wild plant species.

Moreover, it involves the maintenance of explants in sterile and pathogen free environments and is mostly used for asexually propagated seeded species. Scheck et al. (1998a) investigated that infected grapevines grow slower, evidenced by reduced trunk diameter, shortened internodes, reduced foliage and reduced leaf size. It offers an alternative to field gene bank.

The present investigations are, therefore, focused on the establishment of suitable protocol for the in vitro propagation of grape genotypes through tissue culture.

MATERIALS AND METHOD

The explants for in vitro conservation studies were obtained from the field gene bank of IABGR, NARC, Islamabad, Pakistan. Fifteen grape genotypes were used for in vitro conservation (Table 1).

These accessions were previously collected from diverse ecologies including Khyber Pakhtunkhwa and Northern Areas and have been used for establishing the field gene bank at NARC, Islamabad. Actively growing buds of the spring season and newly grown shoots were excised from the plant and immediately dipped in water to avoid dessication before bringing them in to the lab. The shoot apices were trimmed and placed in a glass jar under a running tap water

Table 1. Accession number and name of grape genotypes

S. No.	Accession No.	Accession Name
1	019965	Gang Agoon Sufaid
2	019966	Gang Agoon Kala
3	019967	Alighar Agoon
4	019968	Logistan Agoon
5	019969	Nar Agoon
6	019970	Sanosir Agoon
7	019971	Biyo Agoon
8	019974	Kishmish Kala
9	019975	Harkar Agoon
10	020017	Dakh-1
11	020019	Dakh-2
12	020024	Dakh-3
13	020025	Dakh-4
14	020026	Dakh-5
15	020028	Angoor

for 30 min. They were trimmed to a three node sized segments and worked out further under clean bench for aseptic operation after they were treated with 0.05% mercuric chloride ($HgCl_2$) for 1, 2 and 3 min, respectively, for surface disinfection. After surface disinfection, they were rinsed for 2-3 min in sterile water then cultured on ¾ strength MS media containing 2mg/l BAP (Kashif et al., 2005).

Composition, Sterilization and Dispensing of Media

The germplasm were produced on 75% MS salts which consists of standard concentration of sucrose and vitamins and a multitude of hormone regime for in vitro culture development. All these were collected from Sigma and Wako Chemical Company. Initially the test tubes were sterilized in autoclave at 121°C for 9 min.

Establishment of In vitro Culture

Studies were conducted to investigate the in vitro response of duration of $HgCl_2$ (surface disinfectant) on different culture establishment (mortality, viability and contamination) and growth related traits such as shoot length and number of nodes per plant of different grape accessions. It is most important that the nodal parts must be surface sterilized (Abbot, 1977). Surface sterilization was performed under aseptic conditions of clean bench (Hitachi). After disinfection, these explants were transferred on to a medium of defined composition under aseptic conditions. The cultures were incubated in the growth room (20 ± 2°C), illuminated with 1000-2000 lux of light, maintained under a photoperiod of 16 h and data were recorded after 4-6 weeks.

After 4-6 weeks, viability, mortality, contamination, number of nodes and shoot length per plant were calculated. Number of the replicates was different for each experiment ranging from 3-10 in Completely Randomized Design (CRD). These parameters were used to analyze the disinfection of culture with different durations of treatment of explants with mercuric chloride (1, 2 and 3 min). The data thus collected were subjected to statistical analysis (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

It was observed that the viability of culture increased with the increased duration of treatment with the disinfectant in all the accessions (Table 2 and 3). Previously it has been studied that grapes in spite of a woody nature have no response to Woody Plant Medium (WPM). Tehrim and Sajid (2011) found that they responded well on 75% MS salts as compared to 100%. However, 100% viability occurred in Accession No. 020017 (Dakh-1) when treated for 3 min with $HgCl_2$, while minimum viability occurred in Accession No. 019967 and Dakh-4 when treated for 1min and 2min, respectively with $HgCl_2$ (Table 2). However, it varied from accession to accession that might be due to reason that explants were taken from field grown plants, which may be infected with the pathogens to varying extent. Dalal et al. (1999) also concluded that source of explants and the cultivars both had significant effect on the culture

Table 2. Data on various parameters affected by treatment of $HgCl_2$ at different time duration

Accession No.	Disinfection time (min)	Viability (%)	Contamination (%)	Mortality (%)	Shoot length (cm)	No. of nodes plant^{-1}
019965 (Gang Agoon Sufaid)	1	30	70	70	2.90	3.0
	2	30	30	70	2.60	3.0
	3	40	20	60	2.10	2.0
019966 (Gang Agoon Kala)	1	20	80	80	2.40	2.5
	2	00	100	100	2.30	2.0
	3	30	70	70	2.10	2.0
019967 (Alighar Agoon)	1	10	80	90	3.78	3.8
	2	20	80	80	3.20	3.4
	3	20	10	80	2.80	2.5
019968 (Logistan Agoon)	1	30	30	70	3.50	3.7
	2	30	20	70	2.98	3.0
	3	40	00	60	2.25	2.6
019969 (Nar Agoon)	1	60	20	40	3.00	2.5
	2	50	10	50	2.80	2.3
	3	60	00	40	2.10	2.0
019970 (Sanosir Agoon)	1	00	90	100	2.40	2.3
	2	20	60	80	2.95	2.9
	3	50	50	50	2.00	1.0
019971 (Biyo Agoon)	1	30	50	70	2.95	2.6
	2	30	50	70	3.70	3.0
	3	40	60	60	3.15	2.7
019974 (Kishmish Kala)	1	20	70	80	2.00	1.0
	2	20	30	80	2.97	2.0
	3	40	30	60	2.97	2.0
019975 (Harkar Agoon)	1	50	20	50	3.95	3.4
	2	60	20	40	3.22	3.0
	3	80	20	20	3.12	3.0

(Contd.)

Accession No.	Disinfection time (min)	Viability (%)	Contamination (%)	Mortality (%)	Shoot length (cm)	No. of nodes plant^{-1}
020017 (Dakh-1)	1	90	10	10	4.12	3.8
	2	90	10	10	3.65	3.5
	3	100	00	0	3.00	3.0
020019 (Dakh-2)	1	50	20	50	3.40	2.6
	2	30	30	70	3.00	2.0
	3	50	30	50	2.40	1.0
020024 (Dakh-3)	1	30	70	70	3.97	3.2
	2	70	20	30	3.25	2.5
	3	80	10	20	3.25	2.0
020025 (Dakh-4)	1	40	30	60	3.70	3.4
	2	10	10	90	3.35	3.3
	3	30	10	70	2.95	2.0
020026 (Dakh-5)	1	70	10	30	3.40	3.4
	2	70	10	30	3.15	3.0
	3	80	00	20	3.00	3.0
020028 (Angoor)	1	50	30	50	3.60	3.5
	2	50	30	50	3.50	3.1
	3	70	20	30	1.60	1.0

survival.

Similarly maximum contamination of 100% occurred in the Accession No. 019966 (Gang Agoon Kala) when treated for 2 min, while no contamination was recorded in Accession No. 020017 (Dakh-1) and Accession No. 020026 (Dakh-5) treated for 3 min (Table 2). In all the accessions, contamination was generally higher in the explants treated for shorter duration with disinfectant. Al-Da-Silva et al. (2000) also reported significant differences mainly concerning shoot and root development and number of nodes among five varieties of grapevine root stocks.

Due to minimum viability and maximum contamination in Accession No. 019966 (Gang Agoon Kala), its mortality was also higher as compared to other accessions. While minimum mortality recorded in Accession No. 020017 (Dakh-1) because of its maximum viability and minimum contamination. The mortality increased or decreased randomly and depicted no association with the duration (Table 2). These results are in line with the findings of Tehrim and Sajid (2011) who reported

that mortality did not depend upon the duration of disinfectant treatment as no mortality was obtained in the shortest duration of treatment.

Maximum shoot length (4.12 cm) and number of nodes plant^{-1} (3.8) was observed in the Accession No. 020017 (Dakh-1) when treated for 1 min (Table 2). Whereas minimum shoot length (1.60 cm) and number of nodes plant^{-1} (1.0) was found in Accession No. 020028 (Angoor) with 3 min treatment (Table 2).

Taken together, 450 explants were cultured in this study out of which 197 explants produced viable cultures after incubation. Whereas 253 explants were dead, perhaps due to toxicity of the disinfectant, 152 explants exhibited incidence of contamination, indicating the ineffectiveness of the disinfectant for these explants (Table 3). The variability in viability, contamination incidence and mortality incidence among each accession may be explained by the differences in explants tenderness, hardiness, growth stage, initial contamination load and also handling variability from time to time as these cultures were made on different days due to lengthy protocols. Only 60-90 explants could be handled every day.

It is thus concluded that this optimization protocol can be useful in fine-tuning the dose and duration of the treatment of explants with the surface disinfectant - mercuric chloride (Hg Cl_2). Maximum viability and good growth in grape genotypes, when grown through tissue culture, can be obtained by treating with mercuric chloride for 3 min. This investigation will be helpful in having an impact on the conservation of grape germplasm and movement for future improvement strategies.

Table 3. Effects of three different time durations of $HgCl_2$ treatments on growth parameters of grape explants

Parameter	Disinfection time (min)			
	1	2	3	Total
Viability	56	60	81	197
Contamination	71	49	32	152
Mortality	25	41	35	101
Total	152	150	148	450

LITERATURE CITED

Abbot, A. J. 1977. Propagating temperate woody species in tissue culture. Sci. Hort. 28: 155-200.

Al-Da-Silva, P. Hariscain, N. Ollat, J. P. Doazan, A. Bougquet, and J. M. Boursiquot. 2000. Comparative in vitro development of five grapevine root stock varieties and mutant from the cultivars 'Gravesac'. Proc. of the 7th Intl. Symp. on grapevine genetics and breeding. Montpelljer, France Acta Horticulturae. 528: 351-357.

Dalal, M. A., C. K. Sahni, A. A. Khan, K. Surinder, and S. Kumar. 1999. Effect of explants source and stock plant treatment on presenting total phenols and culture nutrient of grapewine in vitro. Appl. Bio. Res. 1(2): 95-98.

GoP, 2010. Agriculture Statistics of Pakistan. Economic Wing, Ministry of Food and Agriculture, Government of Pakistan, Islamabad.p.98

Kashif, M., G. M. Sajid, and R. Anwar.

2005. Effect of duration of $HgCl_2$ treatment on culture viability, contamination and mortality of various accessions of grapes. Sarhad J. Agric. 21(1): 25-29.

Purohit, S. S. 2003. Plantation crops improvement through biotechnology. Agricultural Biotechnology. 2nd edn. CBS Publishers, Dehli. p. 445-453.

Sagawa, Y. 1984. Colonal propagation: Orchids. In: I.K. Vasil. (ed.). Cell culture and somatic cell genetics of plant. Academic Press, Tokyo. 11: 61-67.

Sagawa, Y., and I. T. Kunisaki. 1982. Clonal propagation of orchid by tissue culture. In: A. Fujiwara (ed.). Plant tissue culture. Maruzen, Tokyo. p. 683-684.

Sajid, G. M., S. Siddique, M. Ishtiaq, I. Haq and R. Anwar. 2003. In-vitro conservation laboratory manual. PGRI, NARC, Islamabad. p. 1.

Scheck, H. J., S. J. Vasquez, W. D. Gubler, and D. Fogle. 1998a. First report of black-foot disease, caused by *Cylindrocarpon obtusisporum* of grapevine in California. Plant Dis.82:448.

Scheck, H. J., S. J. Vasquez, W. D. Gubler, and D. Fogle. 1998b. Three *Phaeoacremonium* spp. cause young grapevine decline in California. Plant Dis. 82:590.

Shuji, N., S. Akira, A. Yoshihiko, and M. Tsunetomo. 1992. Cryopreservation of Asparagus (*Asparagus officinalis* L.) embr-yogenic cells and subsequent plant regeneration by a simple freezing method. Cryo-Letters. 13: 379-388.

Steel, R. G. D., and J. H. Torrie. 1980 Principles and procedures of statistics: A biometrical approach. 2nd edn. McGRaw Hill, Book Co. New York. 663 p.

Tehrim, S., and G. M. Sajid. 2011. In vitro establishment, conservation and its Implications for grape germplasm biodiversity. Romanian Biotechnological Letters, 16(6): 6781-6789.