In vitro Preservation of Grapevine (*Vitis vinifera* L.) Muscat of Alexandria and Black Monukka Cultivars as Genetic Resource

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**Abstract:** The objective of this study was to evaluate *in vitro* storage of grapevine “Muscat of Alexandria and Black Monukka” cultivars under slow-growth conditions. Shoot tips were stored at low temperature 15°C for 3, 6, 9 and 12 months. Murashige and Skoog’s medium (MS) supplemented with appropriate concentrations of osmotic agents (ribose and sucrose) at various concentrations (100, 200 and 300 µM/l) or growth retardant, alar (B9; succinic acid 2,2-dimethyl hydrazide) at various concentrations (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l) were tested. After storage duration (3, 6, 9 and 12 months) survival percentage for shoots were evaluated. *In vitro* cultures maintained at 15°C on MS medium containing ribose or alar showed positive affected for preservation the two cultivars. After twelve months of storage, the culture successfully regenerated into new shoots and they were morphologically similar to those of non-stored.

**Key words:** Grapevine % *In vitro* storage % Alar (B9) % Ribose and sucrose % Recovery

**INTRODUCTION**

*In vitro* techniques can be used for the propagation and conservation of rare or endangered species and crop genetic resources in both agriculture and horticulture "either" for the current production of new plants or for short to medium–term storage [1]. *Vitis vinifera* L. (grapevine) is one of the major fruit crop worldwide and is of high economic interest [2]. Grape germplasm is most commonly used in field gen banks, but will face serious problems in the near future due to large amount of space required and high cost of maintenance [3, 4]. An *in vitro* germplasm preservation method could reduce the labor and space requirements of traditional storage methods [5]. This preservation technique also favors to maintain pathogen free plant materials, safely distribution and cultures are not damage by adverse weather conditions [6]. Conservation of plant genetic resource by *in vitro* technology has been done by slow growth procedures or cryopreservation [7, 8]. Slow growth is generally achieved by reducing growth rate through the use of growth retardants such as Alar (B 9) on pear genotypes [9]. Low temperature, [10] and addition of osmotic agents (sucrose, sorbitol, ribose and mannitol [11-13] globe artichoke and banana. Osmotic agents act as a growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the explants [14, 15]. Besides temperature and osmotic agents and growth retardants are also used for *in vitro* germplasm conservation with abscisic acid (ABA) and Alar (B9) [9]. Although research on the development of conservation techniques has been done for numerous plant species, up to now there is no report of methodologies for *in vitro* conservation of grapes. Muscat of Alexandria and Black Monukka cultivars. In addition of osmotic agents or growth retardants to culture media to suppress shoot growth and lengthen subculture duration at normal culture temperatures. This storage technique is generally applicable to wide range of fruit tree genotypes is extending the ordinary subculture duration from few weeks [16] to 6 months [10] on grapes plant and different plant species [13]. Minimal growth storage is very simple technique and has been studied in several laboratories for *Pyrus* germplasm conservation [17, 18]. Barlas and Skene [19] have reported successful storage of 7 species of grapes for periods up to 12 months. After preservation

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through these techniques, cultures can be readily brought back to normal culture conditions at any time to produce plants on desire. Minimal growth methods or preservation of fruit plants are well established [20]. Therefore, in vitro shoot tip culture under minimal growth storage conditions represent reliable mean of fruit tree conservation [9] further application, to use in vitro techniques to store specific genotypes was recognized by several scientists as a way of conserving the genetic resources of such problem crops [21].

The aim of this study is to in vitro preservation of two grapevine cultivars or short-term period through in vitro storage of shoot tips culture by slow growth methods.

**MATERIALS AND METHODS**

**Establishment of Tissue Culture:** Shoot tips of grapevine, Muscat of Alexandria and Black Monukka cultivars were taken and their basal parts were removed to 0.5 cm. Then, shoot tips were washing by running tap water for 24 hours, followed by rinsed five times with sterile antioxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid) to avoid browning of the tissues. Then the explant materials, subsequently were surface – sterilized in different concentrations (0.26, 0.52, 0.78 and 1.05%) of sodium hypochlorite (NaOCl) solution containing three drops of Tween-20 for 5, 10, 15 and 20 minutes. Then, shoot tips were rinsed three times with sterile distilled water. The sterilized shoot tips were used as experimental materials.

**Plant Materials:** In this study, in vitro plants of grape (*Vitis vinifera* L.) Muscat of Alexandria and Black Monukka cultivars were used for in vitro preservation using different method of short to medium term storage. Shoot tip explants of these cultivars were established on Murashige and Skoog (MS) medium [22] supplemented with 30 g/l sucrose, 2.7 g/l phytagel and 2mg/l 6-N benzyl adenine (BA). In vitro preservation minimal growth medium with osmotica (slow growth techniques) and sugar types on survival percentage of grapevine cultivars were studied. Shoot tip explants of both cultivars (0.5 cm long) were excised and taken after 1st subculture and separately cultured in 350 ml culture jars containing 30 ml of full strength MS medium supplemented with 100 mg/l myo-inositol, 2.7 g/l phytagel with any plant growth regulators. Two types of sugar were tested, sucrose and D-ribose at different concentrations (100, 200 and 300 µM/l). Effect of growth retardants, alar (B9) on survival percentage of grapevine cultivars were tested. Excised shoot tips (0.5 cm long) were taken after the 1st subculture and separately cultured in full strength MS medium supplemented with different concentrations of alar at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). All culture were stored at 15±2°C and under at 16 hours of light and 8 hours of darkness photoperiod with cool white fluorescent light intensity of 3000 Lux. Survival percentage of the shoots was recorded every three months during the storage periods (3 to 12 months). Effect of different sugar types and alar (B9) on regrowth rate of Muscat of Alexandria and Black Monukka cultivars after regeneration of shoot tips from twelve months stored cultures. The survived shoot tip explants from Muscat of Alexandria and Black Monukka cvs. cultures were excised (1 cm long) and taken after twelve months of storage then cultured in jars 350 mm filled with 25 ml of full strength MS medium supplemented with 30g/l sucrose, 2.75 g/l phytagel, 2 mg/l BA and 0.1 mg/l IBA. The cultures were incubated at 25±2°C under at 16 hours light and 8 hours dark photoperiods supplied by fluorescent lamp light intensity of 3000 Lux (normal conditions). Recovery percentage and number of proliferated shoots (new proliferated shoots) were recorded after 4 weeks.

**Statistical Analysis:** Experiments were run in completely randomized design and data were statistically analyzed using standard error according to the method described by Snedecor and Cochran [23]. Each treatment consisted of at least 5 jars with three shoots per jar.

**RESULTS AND DISCUSSION**

*In vitro Conservation via Minimal Growth Medium with Osmotica under Storage Temperature (15°C):* Effect of ribose and sucrose concentrations on survival percentage of Muscat of Alexandria and Black Monukka shoot tip cultures during in vitro storage at 15°C. After 3 months storage, data in Table 1 illustrated that the highest survival percentage (98.14) was noticed with Black Monukka cv. compared with Muscat of Alexandria cv. (96.14) without significant differences between them. The specific effect of osmotica showed that insignificant differences among all tested ribose or sucrose levels on survival percentage were existed except control (87µM sucrose) gave the lowest survival percentage (80%). The interaction between the two studied factors (sugars and cultivars) showed that the highest survival percentage (100%) was obtained with all osmotica concentrations without significant differences among
Table 1: Effect of ribose and sucrose concentrations on survival percentage of grapevine (Vitis vinifera L.) Muscat of Alexandria and Black Monukka cultivars culture during in vitro storage under 15°C

<table>
<thead>
<tr>
<th>Sugar types</th>
<th>Ribose</th>
<th></th>
<th></th>
<th>Sucrose</th>
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<th>Mean</th>
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</thead>
<tbody>
<tr>
<td>Cultivars</td>
<td>0.0</td>
<td>100</td>
<td>200</td>
<td>300</td>
<td>100</td>
<td>200</td>
<td>300</td>
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<tr>
<td>Muscat of Alexandria</td>
<td>73 b</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>96.14 A</td>
</tr>
<tr>
<td>Black Monukka</td>
<td>87b</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>98.14 A</td>
</tr>
<tr>
<td>Mean</td>
<td>80B</td>
<td>100 A</td>
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<td>100 A</td>
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<td>After 3 months</td>
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<td>Muscat of Alexandria</td>
<td>53e</td>
<td>93b</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>87 c</td>
<td>67 d</td>
<td>85.71 A</td>
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<tr>
<td>Black Monukka</td>
<td>73 d</td>
<td>100 a</td>
<td>100 a</td>
<td>100 a</td>
<td>93 b</td>
<td>80 c</td>
<td>60 e</td>
<td>86.57 A</td>
</tr>
<tr>
<td>Mean</td>
<td>63 C</td>
<td>96.5A</td>
<td>100 A</td>
<td>100 A</td>
<td>96.5A</td>
<td>83.5B</td>
<td>63.4C</td>
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<td>After 6 months</td>
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<tr>
<td>Muscat of Alexandria</td>
<td>0.0 g</td>
<td>67c</td>
<td>73b</td>
<td>80a</td>
<td>60d</td>
<td>53 e</td>
<td>20 f</td>
<td>50.43 B</td>
</tr>
<tr>
<td>Black Monukka</td>
<td>0.0 f</td>
<td>87 b</td>
<td>87 b</td>
<td>93 a</td>
<td>87 b</td>
<td>73 c</td>
<td>47 d</td>
<td>67.71 A</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0 E</td>
<td>72D</td>
<td>80B</td>
<td>86.5A</td>
<td>73.5C</td>
<td>63E</td>
<td>33.5F</td>
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<tr>
<td>After 9 months</td>
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<tr>
<td>Muscat of Alexandria</td>
<td>0.0 e</td>
<td>33a</td>
<td>27b</td>
<td>33a</td>
<td>27b</td>
<td>13 c</td>
<td>7d</td>
<td>20 B</td>
</tr>
<tr>
<td>Black Monukka</td>
<td>0.0 e</td>
<td>47 c</td>
<td>53 b</td>
<td>73 a</td>
<td>20 f</td>
<td>7 d</td>
<td>0.0 e</td>
<td>28.57 A</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0 F</td>
<td>40 B</td>
<td>40B</td>
<td>53 A</td>
<td>23.5C</td>
<td>10 D</td>
<td>3.5E</td>
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<td>After 12 months</td>
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Means followed by the same letter (s) are not significantly different from each other at 5% level.

them. Meanwhile, Muscat of Alexandria shoot tips cultured on control medium gave the lowest survival percentage (73%) with significant differences among all treatments. After 6 months storage, data in Table 1 showed that the highest survival percentage (86.57%) of Black Monukka cv. compared to Muscat of Alexandria cv. (85.7%). The specific effect of osmotica revealed that ribose at 200 and 300 µM gave hundred survival percentage followed by ribose and sucrose with the concentration (100 µM) gave 96.5 survival percentage and significant differences among all treatments on survival percentage were existed.

Increasing of sucrose concentrations caused decrease in survival percentage of shoots. Meanwhile, control treatment gave the lowest survival percentage (63%). The interaction between the two studied factors cleared that, different concentrations of ribose for Black Monukka cv. and the concentrations 200, 300 µM/L of sucrose for Muscat Alexandria cv. gave hundred percentage. Meanwhile, the lowest survival percentage (53 and 73%) was obtained with control treatment for Muscat of Alexandria and Black Monukka, respectively. After 9 months storage, the highest significant survival percentage (67.71%) was recorded with Black Monukka cv. compared to Muscat of Alexandria cv. which gave 50.43%. The specific effect of osmotica on survival percentage showed that ribose at 300 µM/L gave the highest survival percentage (86.5%). Increasing of ribose concentrations caused a significant increased the survival percentage. Meanwhile, control treatment for both cultivars (Muscat of Alexandria and Black Monukka cvs.) was died. The interaction between the studied factors cleared that Black Monukka cv. with ribose at 300 µM/L recorded the highest survival percentage (93%) followed by Muscat of Alexandria stored on medium contained with 200 µM/L or 100 µM/L ribose or 100 µM/L sucrose for Black Monukka cv. which gave (87%). Meanwhile, control treatment for both cultivars failed to survive. After 12 months storage, data in Table (1) cleared that the highest significant survival percentage (28.57%) for Black Monukka cv. compared to Muscat of Alexandria cv. (20%) was recorded. The specific effect of osmotica on survival percentage showed that ribose at 300 µM/L gave the highest survival percentage (53%) followed by ribose at 100 and 200 µM/L. Increasing of sucrose concentrations from 100 to 300 µM/L decreased survival percentage. Meanwhile, control treatment didn't give any survival percentage. The interaction between the two studied factors cleared that Black Monukka cv. with ribose at 300 µM/L obtained the highest survival percentage (73%). While, Muscat of Alexandria cv. gave 33% survival when stored on medium contained 100 or 300 µM/L ribose (Fig. 1). No, survival percentage for both cultivars on control medium and medium supplemented with 300 µM sucrose were recorded. The osmotic agent, ribose and sucrose significantly affected recovery percentage in both
Fig. 1: Effect of osmotic agent (ribose at 300 µM/l) of grapevine storage for twelve months at low temperature 15°C.

Fig. 2: Recovery of grapevine storage on at low temperature of 15°C and osmotic agent (ribose at 300µM/l) after twelve months.

Table 2: Recovery percentage and number of proliferated shoots of grape cultivars (Muscat of Alexandria and Black Monukka) after twelve months storage under regrowth medium supplemented with osmotic agents at different concentrations

<table>
<thead>
<tr>
<th>Osmotic agent</th>
<th>Recovery (%)</th>
<th>Number of proliferated shoots after recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 µM/l</td>
<td>200 µM/l</td>
</tr>
<tr>
<td>Muscat of Alexandria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>80b</td>
<td>86.7a</td>
</tr>
<tr>
<td>Sucrose</td>
<td>66.7b</td>
<td>73.3a</td>
</tr>
<tr>
<td>Black Monukka</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>80c</td>
<td>86.7b</td>
</tr>
<tr>
<td>Sucrose</td>
<td>73.3c</td>
<td>80b</td>
</tr>
</tbody>
</table>

Means followed by the same letter(s) are not significantly different from each other at 5% level.

cultivars and the interaction between type of carbohydrates and concentrations were not significant (Table 2 and Fig. 2). Microshoots recovery were 78.5% and 86.7% for Muscat of Alexandria and Black Monukka, respectively with no differences in the normal growth and development of shoots at different ribose concentrations. No clear difference between ribose and sucrose on recovery percentage and number of proliferated shoots for stored shoot cultures of grapevine cultivars were observed. However, ribose showed positive effect on the proliferation of recovered shoot cultures.

Carbohydrates strongly affect growth and physiology of plants in all in vitro culture phases, including conservation, as they serve both as carbon sources for cultured tissues and as osmotic regulators in the medium [24]. Sucrose is almost universally used as the most suitable energy source for plant micropropagation. Ribose is a special carbohydrate that is used in the plant for energy production in the cell as it plays a critical role in the production of ATP. Moreover, it comprises the backbone of RNA, a biopolymer that is the basis of genetic transcription. The high concentration of sugar...
may be harmful and cause plant death. Sarkar and Naik [25] reported that 2 or 4% mannitol could enhance survival of plant gremlasm conserved in vitro. However, the lethal concentration seems to be species dependant. The results of the present investigation are in agreement with those reported by Bekheet and Usama [26], who mentioned that the presence of mannitol or sorbitol in culture medium at 40 g/l for each had a retardant effect on the growth and development of globe artichoke cultures. In this respect, replacement of sucrose by ribose allowed the conservation of banana plantlets for 24 months [2]. Effect of alar (B9) concentrations on survival percentage of Muscat of Alexandria and Black Monukka cvs. shoot tips culture during in vitro storage at 15°C. After 3 months storage, data in Table 3 illustrated that survival percentage were 95.5 and 97.5% for Muscat of Alexandria and Black Monukka cvs., respectively (Fig.3 A and B).

As for the specific effect of growth retardant, data revealed that insignificant differences among alar concentrations from 0.1 to 4.0 mg/l which gave 100% survival percentage. Meanwhile, control treatment (without alar) gave the lowest survival percentage (86.5%). The interaction between the two studied factors (cultivar and alar) gave 100% survival percentage from 0.1 to 0.4 mg/l alar. Meanwhile, alar at 0.5 mg/l gave 93% survival percentage for each cultivars while, control treatment (without alar) gave the lowest survival percentage (80%) for Muscat of Alexandria while, Black Monukka gave 93% survival percentage. After 6 months storage, data in Table 3 cleared that the highest survival percentage (72.2%) was noticed with Black Monukka cv. compared to Muscat of Alexandria cv. (70.0%). The specific effect of growth retardant showed that alar at 0.4 mg/l recorded hundred percentages. Also, increasing alar concentrations from 0.1 to 0.4 mg/l increased the survival
Fig. 4: Recovery grapevine cultivars after twelve months of storage at low temperature of 15°C on growth retardant alar (B9).

Table 4: Recovery percentage and number of proliferated shoots for Muscat of Alexandria and Black Monukka cultivars after twelve months storage under alar concentrations supplements with regrowth medium

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Alar Levels (mg/l)</th>
<th>No. of proliferated shoots after recovery</th>
<th>Alar Levels (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Muscat of Alexandria</td>
<td>53.3d</td>
<td>60.0c</td>
<td>66.7b</td>
</tr>
<tr>
<td>Black Monukka</td>
<td>60.0d</td>
<td>66.7c</td>
<td>73.3b</td>
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percentage then decrease suddenly at 0.5mg/l. On the other hand, control medium did not give any response (0.0%). The interaction between the two studied factors cleared that alar at 0.4 mg/l gave hundred survival percentages; followed by alar at 0.3 mg/l gave 93% for both cultivars. However, control medium (free alar) gave 0.0 survival percentage for both cultivars. After 9 months storage, data revealed that the highest survival percentage (57.8%) was recorded with Black Monukka compared to Muscat of Alexandria which gave 57.8% survival percentage. The specific effect of growth retardant showed that alar at 0.4 or 0.3 mg/l recorded the highest significant survival percentage (80 and 76.5%), respectively. Also, increasing alar concentrations from 0.1 to 0.4 mg/l increased the survival percentage then decreased at 0.5 mg/l while, control medium gave the negative value (0.0%). The interaction between the two studied factors cleared that the highest survival percentage (80%) on medium containing 0.4 mg/l alar for both cultivars, were recorded. After 12 months storage, data in Table 3 indicated that survival percentage cultures were sharply decreased as storage duration increased. Also, data revealed that the highest survival percentage (40%) with Black Monukka compared to Muscat of Alexandria was recorded. As for the specific effect of growth retardant (alar), data showed that significant differences among all treatments on survival percentage were existed. The interaction between the two studied factors (cultivars and alar) showed that the percentage of survived shoot (60%) on medium containing 0.4 mg/l alar for both cultivars were recorded. Most of these shoot tips culture were viable enough to convert into healthy shoots on recovery. Generally, data indicated that Black Monukka recorded the highest survival percentage compared with Muscat of Alexandria. However, the highest survival percentage was obtained with alar at 0.4 mg/l for Black Monukka and Muscat of Alexandria cultivars. A decline in survival percentage was recorded there after increasing the duration of storage to 9 and 12 months at 0.4 mg/l alar for both cultivars.

Data in Table 4 showed that the highest recovery percentage and number of new proliferated shoots/explant (80% and 1.36) of Muscat of Alexandria cv. and (65.3% and 1.96 shoots) were noticed with Black Monukka cv. Depending on the concentrations, alar (B9) significantly affected the recovery percentage and number of proliferated shoots for both cultivars (Table 4 and Fig. 4A and B). In general, for both cultivars, an increase in all
values was observed with the increase of Alar concentrations, from 0.1 to 0.4 mg/l. The survival of shoots at 0.4 mg/l Alar was 80% for both cultivars, but the high concentrations of Alar at 0.5 mg/l was a decreased recovery percentage which gave 40 and 46.7% for Muscat of Alexandria and Black Monukka, respectively. For Alar concentrations at 0.3 or 0.4 mg/l showed no significant effects on proliferated shoots were recorded the highest number of shoots 2.1, 2.3 and 2.5, 2.8 shoots/explant for Muscat of Alexandria and Black Monukka cvs., respectively. However, the lowest shoots number (0.0 and 0.8 shoots/explant) at 0.5 mg/l Alar for Muscat of Alexandria and Black Monukka cvs, respectively were recorded. Slow growth is usually achieved by reducing the culture temperature, by modifying culture medium supplements with osmotic agents of Drosophyllum lusitanicum [27] and Podophyllum peltatum plants [28] and growth inhibitors, or by removing growth promoters to reduce the cellular metabolism of the material, striving to maximize the time between subcultures [29-31]. Storage under low temperature is on of the major tissue technique used for preservation of genetic resources [32-34], under such condition, accumulation of unsaturated lipids on the cell membrane would cause cell membrane thickening and retard cell division and elongation [35]. Although, growth retardants have been successfully used for in vitro preservation of different plant species [36]. In Addition Alar (B9) in culture media resulted in reduce survival and regenerated rates survival. Growth retardant was ineffective to suppress growth for in vitro germplasm storage of woody species [9]. Growth retardant (alar) was useful to suppress shoot growth and length subculture duration [37]. Finally, slow growth in vitro preservation using osmotic agents are materials that reduce the water potential of cell. High concentration of osmoticum in the medium cause a negative water potential and reduce the optimal turgor pressure needed for cell division and inhibit growth [38]. The addition of osmotica to the culture has been proved to be efficient in reducing growth and increasing the storage life of many in vitro grown tissues of different plant species [39]. According to the high levels of osmotic agents in the medium would inhibit both callus growth and shoot formation [40]. Sucrose and manitol [41]; sorbitol and mannitol [16], ribose [29] and [42, 43] and 2,2–dimethyl hydrazide (B-995) [44], were reported to be good materials to lengthen the storage life of in vitro grown tissues. Sucrose is a major component most tissue culture media. It functions as both a carbon/energy source and osmotic agent [13]. Sucrose can be used to reduce plant growth in vitro [33]. Carbon sources perform in synthetic pathway of many compounds, acting as building blocks of macromolecules and may control several developmental processes in the cell [45, 46]. Koch [47] demonstrates that sugar control the expression of many plant genes and their connection to metabolic and developmental process is unequivocal. Hence, carbohydrates are of prime importance for in vitro morphogenesis, a high energy requires process [48]. The storage of healthy germplasm enables extended subculture intervals, thus reducing the time needed and cost of maintenances. From the previous results clear that, used of osmotic agents and growth retardants were useful for in vitro preservation of two grapevine cultivars for twelve months at 15°C. Regeneration and length for duration of subculture of shoots were observed successfully and gave healthy shoots.

CONCLUSION

Storage in vitro of grapevine was possible for twelve months under low temperature at 15°C. Using ribose and sucrose as osmotic agents were useful of storage, from pervious results it is clear that, ribose was better than sucrose. Alar (B9) is suitable for conserving Vitis vinifera L. shoot tips and gave complete regrowth ability after twelve months of preservation.

REFERENCES


