Valbona SOTA, Efigjeni KONGJIKA [1]

SLOW GROWTH *IN VITRO* CONSERVATION OF *ZIZYPHUS JUJUBA* MILL

SUMMARY

This study is undertaken to develop a protocol for *in vitro* conservation of *Zizyphus jujuba* Mill. using different methods for mid-term storage. This specie is very important for the pharmacological values of fruits and leaves, and has a very common use in food industry and for its fragrance. For this reason, it would be of interest to use a method available for *in vitro* multiplication and medium-term germplasm conservation, which involves strategies to slow plant growth through chemical and environmental manipulation of *in vitro* conditions. Shoot tips, used as primary explants, were cultured onto MS medium supplemented with MS vitamins, BAP (6-benzilaminopurine) 1 mg l^{-1}, IBA (3-indole bytiric acide) 0.05 mg l^{-1}, 3% sucrose and 0,57% agar-agar. The pH value was established in 5.6. Three different methods of short and mid-term conservation were examined using *in vitro* grown plant cultures: 1- Effect of reduced sucrose and MS salts concentrations; 2- Combination of low temperature and light regime; 3- Absence of phytohormones or growth regulators in the growth media. Maintenance in these conditions reduced microcuttings growth. Were evaluated the survival and regeneration rate for different periods. To test the regeneration of the conserved cultures, they were transferred onto fresh culture medium. The examined methods differed significantly in the survival rate of the explants. The effect of low temperature (4^{o}C) combined with reduced light regime is the most effective method of medium term preservation. The optimal time of conservation without subculture on 4^{o}C was 10 months.

Keywords: *In vitro* conservation, survival, regeneration, *Zizyphus jujuba*

INTRODUCTION

Maintenance and preservation of diversity in cultivated species and wild relatives of crop plants is not only important to breeders for crop improvement programs, but also to human being for fulfilling their nutritional developments.

Zizyphus jujuba (Rhamnaceae) fruit commonly known as jujube or Chinese date is used widely for the treatment of different diseases such as treatment of chronic fatigue, loss of appetite, diarrhea, anemia, irritability and hysteria. The fruits are also believed to possess activities such as anodyne, anticancer, refrigerant, sedative, stomachic, styptic and tonic. The principle chemical constituents of *Zizyphus jujuba* fruit are flavonoids, saponins, tannins,

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vitamins A, B_2, and C, sugars, mucilage, calcium, phosphorus, and iron (www.ibiblio.org, 2009). *Zizyphus jujuba* is reported for anti-complementary (Lee et al. 2004), anti-cancer (Huang et al. 2007), hypoglycemic (Iganacimuthu and Amalraj 1998) and anxiolytic activities (Peng et al. 2000). Furthermore, traditionally, jujube is used prophylactically for liver diseases (Khare, 1995). The fruits are also used in Chinese medicine to strengthen liver function (www.ibiblio.org, 2009).

In vitro culture is an effective method for *ex situ* conservation of plant genetic diversity, allowing rapid multiplication from very little plant material and with little impact on wild populations. There are two types of *in vitro* preservation method used in tissue culture. These include: a) growth retardation (slow growth or minimal growth) method and b) cryopreservation or ultra-low temperature preservation. The first method is used for the preservation of genetic resources for medium terms (from several months to a few years), the latter method is for the preserving for long time (for several decades or longer) (Day and Stacey 2007). These alternate preservation techniques are less costly and safe to conserve germplasm.

For safe preservation, the *in vitro* slow growth storage method was developed and is considered an alternate solution for medium term storage of fruit germplasm (Neveen and Bekheet 2008). The aim of medium term storage is to increase the interval period between subcultures by reducing growth. This might be achieved by the use of modified environmental conditions, modified culture medium, growth retardants, osmotic regulators and/or reduction of oxygen concentration (Kameswara, 2004). Slow growth storage via *in vitro* cultures has been reported in many species (Maqsood et al. 2010).

The present study aims to develop a procedure for *in vitro* mid-term storage of *Zizyphus jujuba* shoots by investigate the effect of reduced concentrations of sucrose in medium containing half-strength Murashige and Skoog (1962) nutrient salts and combination of low temperature and light regime.

MATERIAL AND METHODS

Plant material: collection and disinfection

Cultures of *Zizyphus jujuba* Mill. are established from apical and lateral buds removed from adult field-grown trees. Plant material is obtained by collecting the active explants between January and March, when buds are starting to swell from shoots in dormancy.

Active shoots were cut in two - or three-node sections. The stem sections are washed carefully with water and then are shaken for 5 min. in 70% ethanol, followed by 20 min. treatment with $HgCl_2$ 0.01% and two drops of Tween 20. Finally, stem sections are rinsed three times with sterile distilled water.

Media composition for *in vitro* cultivation

-*Proliferation and elongation medium:* Basal nutrient medium MS (Murashige and Skoog 1962) with thiamine-HCl 0.4 mg l^{-1}, nicotinic acid 0.5 mg

l^{-1}, pyridoxine 0.5 mg l^{-1}, glicine 2 mg l^{-1}, myo-inositol 100 mg l^{-1} was used. The media is supplemented with phytohormones cytokinin 6-benzylaminopurine (BAP) 1 mg l^{-1} and auxin indole-3-butyric acid (IBA) 0.05 mg l^{-1}. The media is enriched with sucrose 3% and agar 0.57%. The pH of the media is adjusted to 5.6. After a month, the developed buds are transferred to fresh media in order to elongate the shoots. Measurements of length of the shoots and leaves number are taken.

-*Subculture and rooting medium:* The subculture medium is similar with the proliferation medium. Microcuttings derived from *in vitro* cultures are placed for rooting in MS medium supplemented with auxin indole-3-butyric acid (IBA) 0.1 mg l^{-1}.

***In vitro* chamber conditions:** The culture in the proliferation stage is grown in the growth chamber at temperature of 25° ± 2° C in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m^{-2} s^{-1}.

For *in vitro* conservation, three different methods of minimal growth are tested:

- *Effect of reduced sucrose and MS salts concentrations*: The cultures are transferred onto ½ MS medium without sucrose and supplemented with the same rate of plant regulators and agar as in the multiplication medium. The incubation conditions are the same as in the multiplication stage.

- *Combination of low temperature and light regime:* The proliferated shoots are incubated at 4^{o}C in dark conditions. The media under these conditions is the same with the multiplication medium

-*Absence of phytohormones or growth regulators in the growth media:* The cultures are transferred onto MS medium without growth regulators or phytohormones and supplemented with the same rate of other components as in the multiplication medium. The incubation conditions are the same as in the multiplication stage

The cultures are stored in these conditions for different periods (3, 6, 10 months) for each method tested. For each method are made three replications and there are at least 15 shoots in each replication. Survival of the cultures is assessed on the basis of criteria as suggested by Reed (1992) as dead and brown shoots are considered as unsurvived while those with vigorous growth and having healthy leaves are considered survived.

Data elaboration: All experiments are repeated at least three times. Experimental data is elaborated by Tucey-Kramer, Student's methods and the analyse of variance (ANOVA) with JMP 7.0 statistical software.

RESULTS AND DISCUSSION

***In vitro* cultivation**

-*Elongation and proliferation stage:* The desinfectation methods results optimal because is the rate of contamination is very low. Contamination rates are about 15 % for primary explants and less than 2% for subcultures. The first signs

of organogenesis induction are observed after two weeks of cultivation under normal conditions of growth in vegetative room. Is not observed the formation of lateral buds in the first stage of organogenesis (Figure 1). Are realised measurements of length of the shoots and leaves number in different periods of proliferation stage (Table 1, Graphic 1a, b).

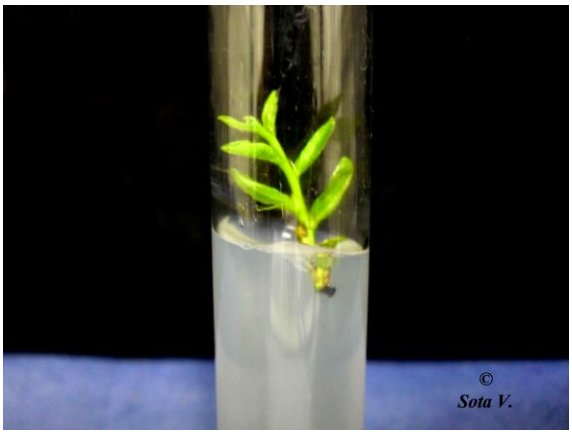
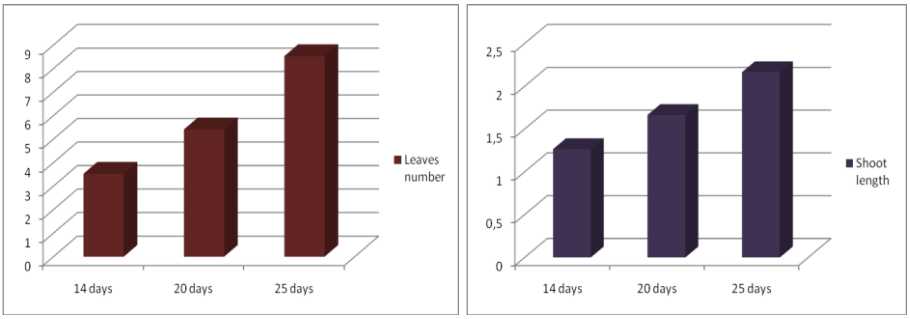


Figure 1. Plantlets of *Z. jujuba Mill.* in the first stage of *in vitro* organogenesis

Table 1. Dynamics of measured parameters during proliferation stage

	14 days	**20 days**	**25 days**
Leaves number	3,5294 ± 0,40327 St dev 1,66274	5,41176 ± 0,55611 St dev 2,29289	8,52941 ± 0,60705 St dev 2,50294
Shoot length	1,26471 ± 0,14347 St dev 0,591546	1,66471 ± 0,16178 St dev 0,667028	2,16471 ± 0,16246 St dev 0,669833



Graphic 1. Dynamics of a) leaves number b) shoot length, during proliferation stage

Other authors (Hossain et al. 2003) have taken positive results during proliferation stage using MS media with a higher concentration of NAA auxin. In

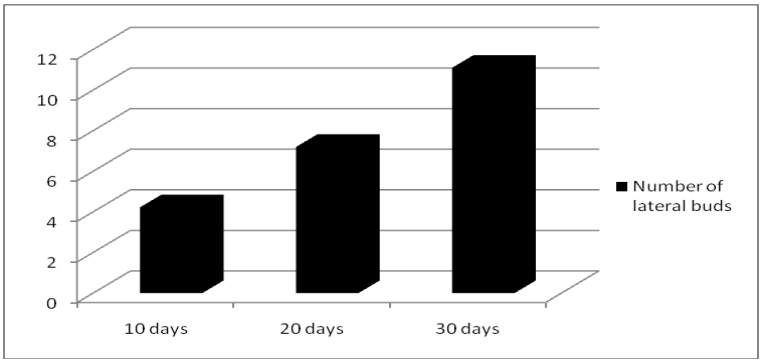
their study, the ratio cytokinin : auxin is low (2,5), whereas in our investigation this ratio is very high (20) in favor of cytokinin BAP, which plays an important role in the first stages of *de novo* bud formation.

-*Multiplication (subculture)* stage: After 25 - 30 days, all explants are inoculated in a fresh media, similar with the proliferation one, and are maintained under the same growth conditions as in the first stage. The obtained plantlets must be subculturated every 2 – 3 weeks in order to obtain a great number of plants for multiplication purposes. Growth dynamics for leaves number and shoot length is almost the same as in proliferation stage, but characteristic of multiplication stage is the formation of a great number of lateral buds (Table 2, Graphic 2, Figure 2).

Table 2. Dynamics of lateral bud's number obtained during different periods of multiplication stage

	10 days	**20 days**	**30 days**
Number of lateral buds	4,2222 ± 0,46481 St Dev 1,39443	7,2000 ± 0,51208 St Dev 1,61933	11,1000 ± 0,76667 St Dev 2,42441

Apical and lateral buds are subculturated independently and each category presents similar growth dynamics for leaves number, shoots length and shoots number as in previous stages. This multiplication system results with high efficiency because the multiplication coefficient is very high.



Graphic 2. Number of lateral buds obtained during multiplication stage

The main factor in the great number of plantlets produced during this stage is the high concentration of cytokinin (BAP) in the media, which stimulates the formation of new shoots by inhibiting the apical dominance (Salisbury and Ross 1992; Taiz and Zeiger 2006).

Characteristic of *Z. jujuba Mill.* plantlets, which is rarey observed in other plants cultivated *in vitro*, is that those resist without subculturing for long periods

(at least 2 months). Even if the leaves begin to become weak and yellow, the plantlets can be regenerated imediately when they are ransferred in a fresh media.

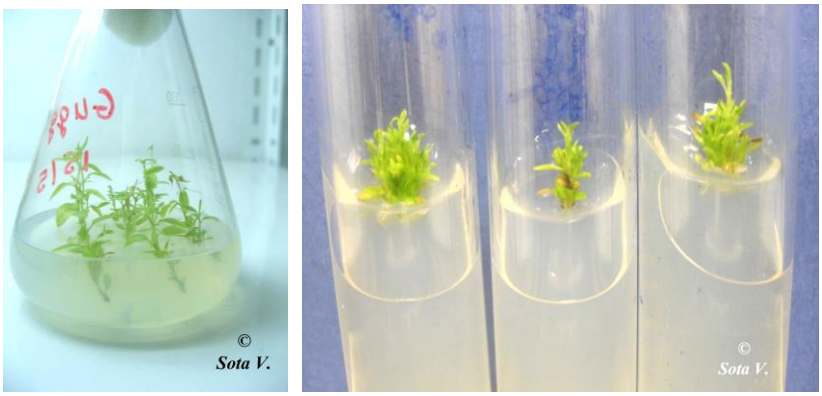


Figure 2. Plantlets of Z. jujuba Mill. during subculture stage

-*Rooting* stage: Shoots obtained during multiplication stages do not present spontaneous rooting in the growth media, so is necessary to transfer the new plantlets in a rooting media. Rooting index is high in presence of auxin (IBA) 0.1 mg l^{-1}. The most important factor is the material inoculated for rooting. Is observed minimal rooting percentage when are placed individual plantlets in the rooting media, and a very high rooting percentage when are placed a group of shoots (Figure 3). Maybe this happen because the contact between the base of the shoot and the media is greater. Characteristic of this stage is the delayed rooting response, after almost 8 weeks of cultivation in the rooting media.

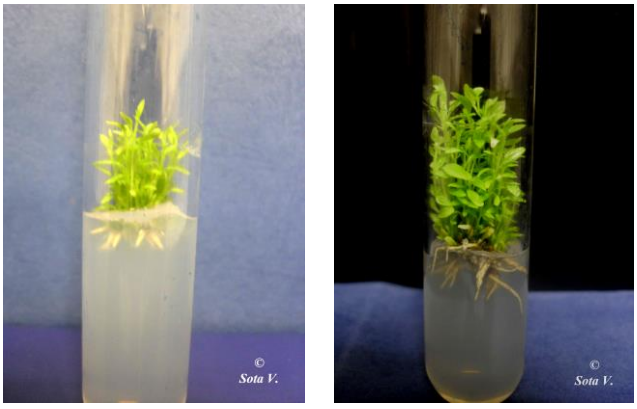


Figure 3. In vitro rooted plantlets of Z. jujuba Mill.

As is reported (Hossain et al. 2003), IBA is considered as “rooting factor”, but positive results in these experiments are observed by using high

concentrations of IBA (1 mg l^{-1}), whereas in our experiment the optimal concentration is 0.1 mg l^{-1}.

***In vitro* conservation**

The response of *in vitro* cultured shoots stored for 3, 6 and 10 months to each method tested is assessed on the basis of survival and regeneration rates. The survival and regenerability rate of shoots for each method tested differed significantly (Table 3, Table 4).

The results obtained for all conservation methods are significantly variable. The maximum survival percentage is recorded in shoots maintained in 4°C in darkness for all periods tested. The least survival is observed in shoots inoculated in hormone free media for all periods tested (Table 3, Graphic 3^a, 3^b). Data presented in Table (3) show that up to 78.6 % of shoot culture remain healthy and green after 10 months storage on 4°C in darkness, while the lowest survival rate (12.6 %) is observed on hormone free medium.

Almost similar pattern is observed for the parameter of regeneration percentage. The maximum regeneration rates were obtained on 4°C in darkness (74.9 % in 10 months) while the lowest regeneration rate (16.6 % in 10 months) is observed on hormone free medium (Table 4, Graphic 4^a, 4^b).

The storage period also has a significant effect on survival and regeneration rates. The highest survival and regeneration rates are recorded for 3-month storage, which are significantly different for the other storage periods. The cultured shoots stored for 10 months presents the greatest decrease in both parameters.

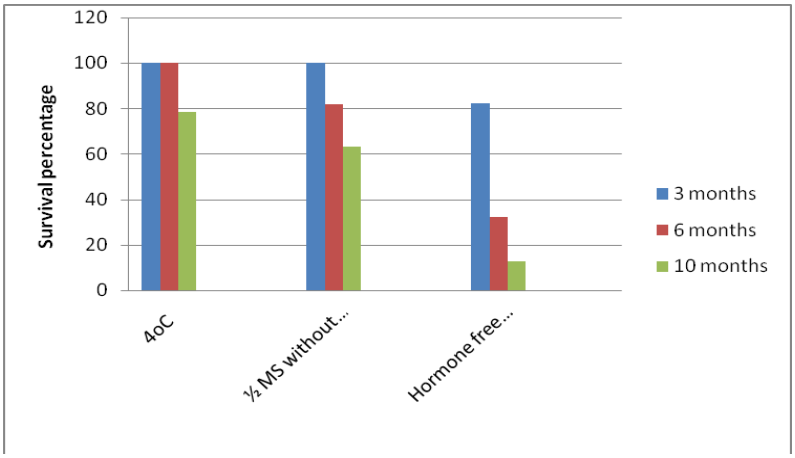
Table 3. Survival of explants conservated in different methods

Survival	**3 months**	**6 months**	**10 months**
4°C in darkness	100 ± 0,00 **A** St. dev. 0,00	100 ± 0,00 **A** St. dev. 0,00	78,6 ± 1,52 **A** St. dev. 0,88
½ MS media without sucrose	100 ± 0,00 **A** St. dev. 0,00	82,0 ± 1,79 **B** St. dev 3,10	63,0 ± 2,30 **B** St. dev. 4,00
Hormone free media	82,3 ±1,76 **B** St. dev 3,05	32,3 ± 1,85 **C** St. dev. 3,21	12,6 ± 1,20 **C** St. dev. 2,08

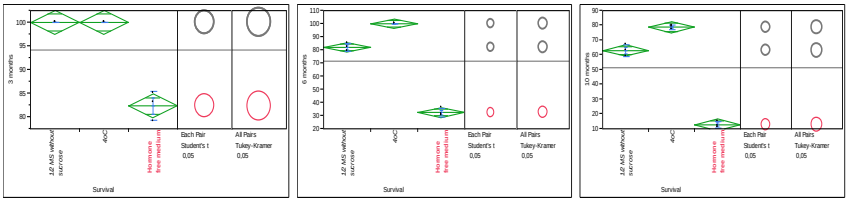
Table 4. Regenerability of explants conservated in different methods

Regenerability	**3 months**	**6 months**	**10 months**
4°C in darkness	100 ± 0,00 **A** St. dev. 0,00	97,0 ± 0,57 **A** St. dev. 1,00	74,9 ± 0,98 **A** St. dev. 1,70
½ MS media without sucrose	96,4 ± 1,63 **A** St. dev. 2,83	77,9 ± 1,62 **B** St. dev 2,81	46,6 ± 2,02 **B** St. dev. 3,51
Hormone free media	91,6 ± 1,76 **B** St. dev 3,05	58,0 ± 2,08 **C** St. dev. 3,60	16,6 ± 0,88 **C** St. dev. 1,52

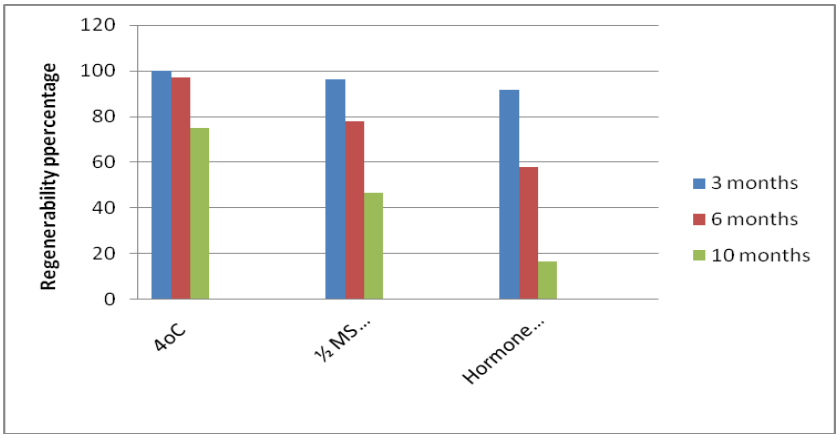
Note: *The values not connected by the same letter are very different between them*



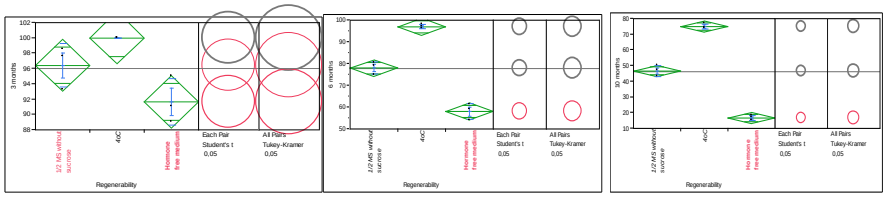
Graphic 3ª. Survival of explants conservated for different periods in different methods



Graphic 3ᵇ. Oneway Analysis for Survival Parameter in different methods of slow growth for three periods tested



Graphic 4ª. Regenerability of explants conservated for different periods in different methods



Graphic 4[b]. Oneway Analysis for Regenerability Parameter in different methods of slow growth for three periods tested

As far as duration of preservation is concerned, 3 and 6 months of storage periods are proved better than longer durations for the two other conservation methods tested. With increase in storage period, survival rate as well as regenerability is reduced significantly. In the cultures, which are stored for the period of 10 months, their survival and regenerating percentage remained below 60 percent. The present investigation revealed that the higher survival rates had positive effect on regeneration percentage of the shoots.

Low temperature has been successfully applied to *in vitro* cultures of various plants species for short and medium term storage. Tahtamouni and Shibli (1999) preserved micro shoots of wild pear (*Pyrus syriaca*) through slow growth (low temperature) technique. In previous study, temperature in the range of 5 to 10^{o}C has been found suitable for short term *In vitro* storage of meristem cultures of several temperate species (Lundergan and Janick 1979).

In the present study, the most effective method of mid term conservation resulted the conservation at low temperature in darkness for different periods (3, 6 and 10 months). These results confirmed the findings of other workers who reported that meristem cultures of pear (Wanas et al. 1986; Bell and Reed 2002), apple (Lundergan and Janick 1979) and apple rootstocks (Orlilkowska, 1992; Negri et al. 2000) can be stored *in vitro* at low temperatures.

Therefore, it is concluded that the shoot tips *Zizyphus jujuba* Mill. can successfully be preserved *in vitro* by lowering culture temperatures for short to medium term storage.

CONCLUSIONS

Micropropagation result a suitable method for obtaining a large number of plants of *Z. jujuba* Mill. The cytokinin BAP in a concentration of 1 mg/l results optimal for this purpose. Rooting index is high if is used the auxin IBA in a concentration of 0.1 mg/l. Maximum survival and regeneration rates were obtained during conservation on 4^{o}C in darkness Conservation in ½ MS media without sucrose results optimal for 3 and 6 months, whereas conservation in hormone free media results optimal only for 3-month conservation period. With increase in storage period, survival rate as well as regenerability was reduced significantly.

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IN VITRO KONZERVACIJA VRSTE *ZIZIPHUS JUJUBA* MILL., (KINESKE URME-IGLICE)

SAŽETAK

Ovo istraživanje je realizovano sa ciljem uspostavljanja protokola za *in vitro* konzervaciju iglice (*Ziziphus jujuba* Mill), primjenom metoda srednjoročne konzervacije. Ova vrsta je veoma važna zbog farmakološke vrijednosti plodova i lišća, i veoma je zastupljena u prehrambenoj industriji kao i zbog svog mirisa. Iz tog razloga, bilo bi od interesa koristiti metod za *in vitro* multiplikaciju i srednjoročnu konzervaciju germplazme, koji obuhvata strategije za usporavanje rasta biljke putem hemijske i ekološke manipulacije *in vitro* uslovima. Vrhovi izdanaka, korišteni kao primarni eksplanti, su izloženi u MS medijumu srednje obogaćenom MS vitaminima, 1 mg l^{-1} BAP 6-benzilaminopurina, 0.05 mg l^{-1} IBA, indol-3-indole buterne kiseline, 3% saharoze i 0,57% agar-agar. Ustanovljena pH vrijednost bila je 5.6. Ispitane su tri različite metode kratkoročne i srednjoročne konzervacije *in vitro* uzgajanih biljnih kultura: 1 - Uticaj redukcije saharoze i MS koncentracije soli, 2 - Kombinacija niske temperature i režima svijetla, 3 - Odsustvo fitohormona ili regulatora rasta u sredini za uzgajanje. Održavanje u ovim uslovima usporilo je rast mikro izdanaka. Procjenjivana je stopa opstanka i regeneracije u različitim periodima. Da bi se testirala regeneracija očuvanih kultura, prebačene su u svježu sredinu. Ispitivane metode su različito uticale na stopu preživljavanja eksplantata. Efekat niske temperature (4^oC) u kombinaciji sa smanjenim režimom svijetla je najefikasniji metod srednjoročne konzervacije. Optimalno vrijeme konzervacije bez subkulture na 4^oC je 10 mjeseci.

Ključne riječi: *In vitro* konzervacija, opstanak, regeneracija, *Zizyphus jujuba* Mill.