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Two-Step Culture Efficiently Improving Shoot Regeneration from in Vitro Leaf Explants of *Zizyphus spinosus* Hu

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Abstract: A highly efficient two-step culture protocol was developed for adventitious shoot regeneration from in vitro leaf explants of sour jujube (Zizyphus spinosus Hu.). In vitro shoots of sour jujube were selected as materials. Expanding young leaves were selected as explants. The effect of basal medium, thidiazuron concentration and culture protocol on shoot regeneration was examined. Results showed that two-step culture was better than one-step culture. First, leaf explants were cultured on the basal medium supplemented with thidiazuron (TDZ) and indole-3- acetic acid (IAA) for callus induction and adventitious buds initiation under dark culture, and then subcultured on the basal medium supplemented with IAA and gibberellic acid (GA3) for shoot buds elongation. The optimal media for shoot buds formation and elongation were WPM medium containing 0.5 - 1mg l-1 TDZ and 0.1 or 0.5 mg l-1 IAA and WPM medium containing 0.1 mg l-1 IAA and 0.5 mg l-1 GA3 separately. The effect of light on shoot regeneration was also examined. The adventitious buds regeneration was observed when leaf explants were cultured in the light, but in this case, the buds were smaller buds directly formed from leaf explants, and couldn't further develop and elongate to form shoots. The two-step regeneration protocol may be applicable to the improvement of this crop by genetic engineering in the future.

Keywords: Adventitious Buds, Bud Elongation; Direct Regeneration, Sour Jujube

1. Introduction

Sour jujube (*Zizyphus spinosus* Hu.) from *Rhamnaceae* family was a wild fruit crop originated from China, it has many different varieties distributed in different area in whole China [10]. Being high resistant to cold and drought, tolerant to saline-alkali, and easily rooting, sour jujube is a good rootstock of cultivated jujube (*Zizyphus jujuba* Mill) [9, 14], also an important forestation crop in mountain and hill area [8, 20]. Having long blossoming period, is a good honey source plant of bees [8]. Being rich in vitamin C and minerals in fruit and seed, sour jujube also is traditional component of Chinese herb [6, 16, 27]. But because they grew mainly in wild or semi-wild state at mountain or hill, they are susceptible to infection of phytoplasma, a disease caused by a phloem-inhabiting, cell wall-less bacterium. This disease was transmitted from diseased plant to healthy plant around by insect vector [28]. Tens of millions of trees were damaged by phytoplasma each year in China, caused large economic loss [15, 25]. Control of phytoplasma disease is very difficult in jujube because of lack of efficient curative methods and plant varieties of tolerant or resistant to phytoplasma.

Artificially engineered resistance resulting from gene manipulation has opened a new strategy for plant disease control. The application of genetic engineering techniques needs an efficient *in vitro* regeneration system. A few cultivars of jujube shoot regeneration had been reported, such as Hami jujube [4, 26] and. Luozao 3 jujube [23]. Shoot regeneration from sour jujube leaf explants was also previously reported [21, 22], but the regeneration rate and shoot number per leaf explants were low. Sour jujube has still been considered to be a recalcitrant for regeneration *in vitro*. The aim of the present study was to optimize an *in vitro* regeneration protocol for sour jujube, to facilitate genetic transformation for this important rootstock of jujube.

2. Method

2.1. Plant Material and Culture Conditions

The fruits of Taishan sour jujube were collected from healthy trees (which was confirmed by PCR analysis) on Mountain Taishan. The kernels were sterilized with 0.1% $HgCl_2$ for 12 min, then rinsed in sterilized water. The seeds were removed from cracked kernels and germinated on hormone-free MS medium (Murashige and Skoog, 1962) at 25°C ± 2°C under cool-white fluorescent tubes with a photoperiod of 16-h light (40μmol$m^{-2}s^{-1}$) and 8-h darkness. Shoots were cut from seedlings and transferred to micropropagation medium to proliferate to obtain enough shoot cultures.

2.2. General Strategy for Shoot-Bud Induction

Expanding young leaves were excised from the top-portion of 3- to 4-week-old in vitro-cultivated shoots, wounded by several transverse cuts across mid-rib, and placed horizontally with abaxial side up onto various shoot-bud induction media (Table 1). All treatments were incubated in the darkness for 4 weeks before transferring to photoperiod condition, with the exception of light experiment.

2.3. Light Experiment

In a separate experiment, all explants were cultured on induction medium I5 for two weeks, then transferred to expression medium II (II1-II4). They were kept in different light conditions: photoperiod condition was 16 h light and 8 h dark; first one or two weeks in the dark, then transferred to photoperiod condition.

2.4. The Protocols of Shoot-Bud Regeneration

The protocols of shoot-bud regeneration included one-step regeneration: leaf explants cultured on induction media (Media I) with TDZ and IAA (Table 1) continuously; and two-step regeneration: first cultured on induction media (media I) for two or four weeks, then transferred to the expression media (media II) with IAA and GA_3 (Table 1).

Table 1. *Composition of induction media (Media I) and expression media (Media II) used for shoot-bud regeneration from leaf explants of Taishan sour jujube.*

Media No.	Media I*	Media No.	Media II
I1	WPM + 0.1 TDZ + 0.1 IAA	II1	MS + 0.1 I AA + 0.5 GA_3
I2	WPM + 0.1 TDZ + 0.5 IAA	II2	MS + 0.1 IAA + 5.0 GA_3
I3	WPM + 0.5 TDZ + 0.1 IAA	II3	WPM + 0.1 IAA + 0.5 GA_3
I4	WPM + 0.5 TDZ + 0.5 IAA	II4	WPM + 0.1 IAA + 5.0 GA_3
I5	WPM + 1.0 TDZ + 0.1 IAA		
I6	WPM + 1.0 TDZ + 0.5 IAA		
I7	MS + 1.0 TDZ + 0.5 IAA		

*: MS: Murashige and Skoog medium, 1962; WPM: woody plant medium (Lloyd and McCown, 1981)

2.5. Statistical Analysis

For each treatment at least three petri dishes were prepared as a one replicate, each dish containing ten explants. Each treatment was repeated three times. The data processing was done by DPS v3.01. Effect of treatments was tested by analysis of variance and differences among means were tested by LSD at 5% level of probability.

3. Results and Discussion

3.1. The Effect of Culture Methods on Adventitious Bud Formation

The frequency of adventitious bud formation when leaf explants cultured continuously on induction media (media I) increased with increasing concentration of TDZ (Table 2), the concentration of TDZ had a significant positive effect on

Table 2. *Adventitious bud regeneration when Taishan sour jujube leaf explants cultured on induction media I continuously for 6 weeks.*

Media No.	Regeneration frequency%*	Shoot bud growth
WPM + 0.1 TDZ + 0.1 IAA	15.5 ± 1.5 d	normal buds
WPM + 0.1 TDZ + 0.5 IAA	18.3 ± 8.5 cd	normal buds
WPM + 0.5 TDZ + 0.1 IAA	35.8 ± 4.4 b	normal buds
WPM + 0.5 TDZ + 0.5 IAA	32.0 ± 8.0 bc	normal buds
WPM + 1.0 TDZ + 0.1 IAA	56.0 ± 9.5 a	normal buds
WPM + 1.0 TDZ + 0.5 IAA	61.0 ± 11.6 a	normal buds
MS + 1.0 TDZ + 0.5 IAA	57.7 ± 14.0 a	hyperhydric buds

*: the mean ± SD of three replicates with a total 90 explants per treatment. The same letter in the column denotes no significant difference at $p < 0.05$ by LSD test.

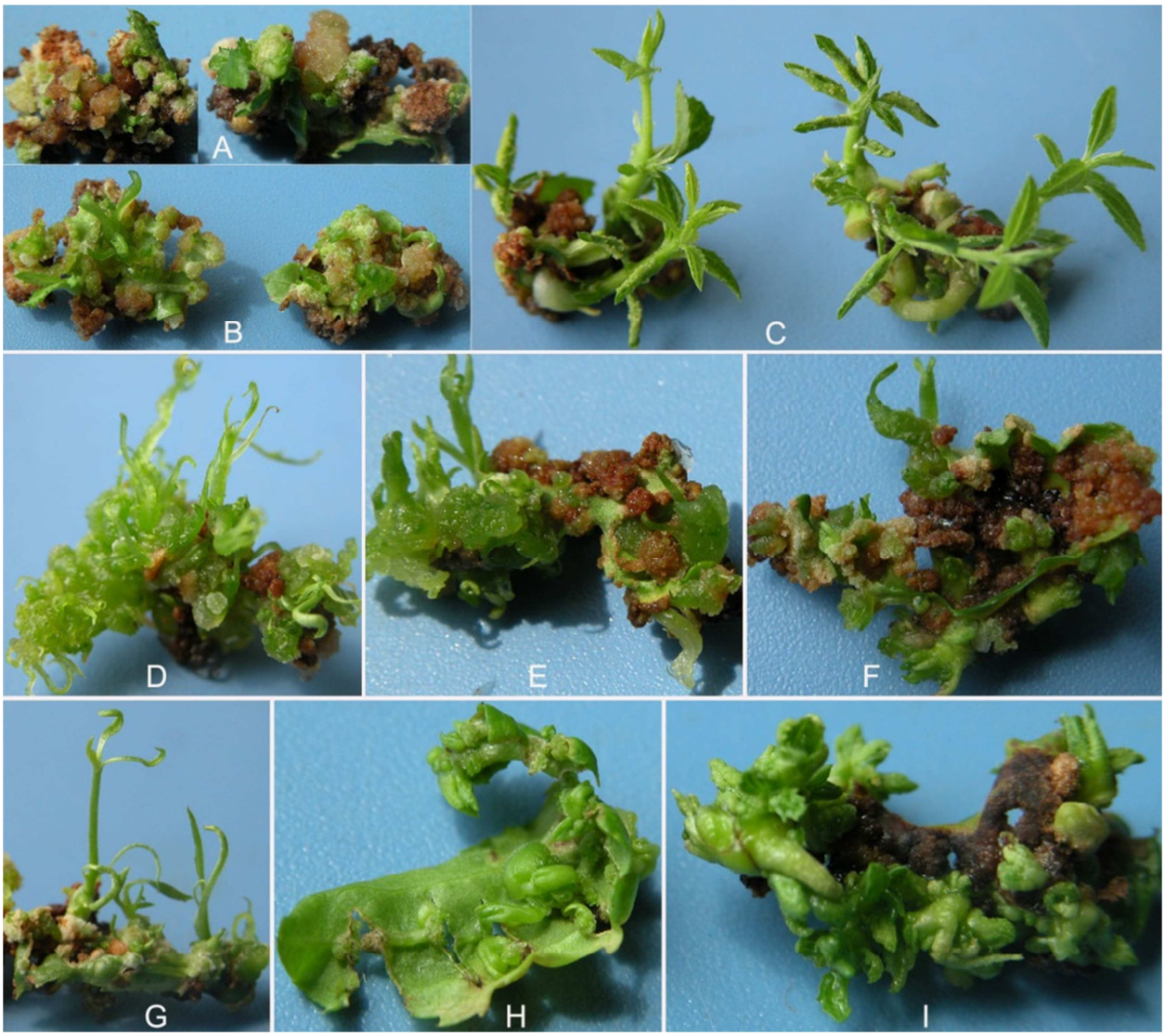

Figure 1. *Adventitious bud regeneration from leaf explants of Taishan sour jujube at different culture conditions.*

A-G: under 4 weeks dark culture. A: inhibited adventitious buds (non-hyperhydric) elongation on medium I (I1-I6) (WPM) continuously; B: inhibited adventitious buds (hyperhydric) elongation on medium I7 (MS) continuously; C: adventitious shoot elongation after 5 weeks (first culture 2 weeks on medium I5 then subcutured 3 weeks on medium II3); D and E: hyperhydric shoots or buds regeneration after 5 weeks from leaf culture, D: first 2 weeks on medium I (I3 – I6) (WPM) then 3 weeks on medium II1 (MS); E: first 2 weeks on medium I7 (MS) then 3 weeks on medium II1 (MS); F: non-hyperhydric buds regeneration after 5 weeks first 2 weeks on medium I7 (MS) then 3 weeks on medium II3 (WPM); G: shoots with thin and long stem internodes from medium II4 (WPM containing higher concentration GA_3 5 mg l^{-1}); H: adventitious buds direct regeneration in photoperiod condition; I: adventitious buds direct regeneration first on medium I5 2 weeks in dark then on medium II3 in photoperiod.

Regeneration frequency. The regeneration frequency was significantly different. among different concentrations of TDZ. But the auxin IAA had no evidently effect on regeneration frequency, there was no significantly difference between 0.1 mg l^{-1} and 0.5 mg l^{-1} IAA at the same concentration of TDZ. The adventitious buds obtained from all media composition maintained buds and couldn't elongate (Figure 1, A and B) to develop into adventitious shoots no matter how long they were kept. The inhibition of shoot elongation was possibly due to TDZ toxicity, as has been reported in many plant species [1, 7, 17, 24], this inhibition was generally overcome by transferring shoot cultures to medium without [7] or with reduced TDZ [1], or other cytokinin [17]. Media I6 and I7 with the same concentration of TDZ and IAA, only basal medium was different, regeneration frequency was no significant different between medium I6 and I7. This result was different from winter jujube that regeneration frequency reached 76.2% on WPM, and only 10.4% on MS [5]. But adventitious buds produced from leaf explants of Taishan sour jujube were different when the basal medium was different. Adventitious buds were normal on basal medium WPM (I1-I6) and were hyperhydric on basal medium MS (I7). The reason of hyperhydric shoots production probably because higher ammonium nitrate or nitrogen content in MS than in WPM (data not shown). The results were similar to previously report that the percentage of hyperhydric shoots were increased with the increases in medium ammonium nitrate content in serviceberry [2], and the reversion of hyperhydric four-wing

saltbush shoots to normal shoots was significantly improved when the removal of ammonium nitrate from the medium formulation [18].

In a separate study, leaf explants were cultured on media I for 2 or 4 weeks, then transferred to media II without TDZ but with IAA and GA_3, the frequency of adventitious buds regeneration was significantly improved (from 56% to 100%, depending on different medium II) (Figure 2), with the exception of media I1 and I2. In this case, large number of buds developed into shoots (Figure 1, C). The results showed that, in the case of sour jujube, two-step culture was necessary to induce adventitious shoot formation from leaf explants. Regeneration frequency presented here was higher than previously reported that one-step culture (WPM with TDZ and NAA) in the same cultivar (regeneration frequency only 47.5%) [21-22]. However, the basal medium composition of media II influenced the shoot bud quality. When medium I was the same, the buds or shoots obtained were hyperhydric and abnormal (Figure 1, d and e) if the basal medium of subculture media II was MS (II1 and II2), independent of the medium I (I1 to I7). And the buds or shoots obtained were healthy and normal (Figure 1, c and f) when the basal medium of media II was WPM (II3 and II4), independent of the media I (I1 to I7). The results revealed that the basal medium composition of medium II affect adventitious bud elongation. Therefore, the optimal basal media of medium I and medium II both were WPM. When the medium I was MS, the shoot buds either remained buds (medium II was WPM, Figure 1, F) or elongated to develop into hyperhydric shoots (medium II was MS, Figure 1, E)

When medium I and medium II both were WPM, morphological characteristics of adventitious shoots were different between medium II3 containing 0.5 mg l^{-1} GA_3 and II4 containing 5.0 mg l^{-1} GA_3. Adventitious shoots showed thin stem and narrow leaves (Figure 1, G) on II4 and showed thick stem and broad leaves on II3 (Figure 1, C) (data not shown). The results showed that 0.5 mg l^{-1} GA_3 was more effective for normal and healthy shoot growth than 5.0 mg l^{-1}. GA_3

3.2. Effect of the Duration of Leaf Explants on Media I on Shoot Regeneration

The regeneration frequency was different between 2 weeks and 4 weeks culturing on media I before subculture on media II (Figure 2). At the lower TDZ concentration 0.1mg l^{-1} (medium I1 and medium I2), the regeneration rates were low and were not significantly different between two weeks (Figure 2, A) and four weeks (Figure 2, B), independent of what medium II was. At 0.5 mg l^{-1}TDZ, the regeneration rates were higher for 4 weeks culture on media I (Figure 2, B) than that for 2 weeks culture on media I (Figure 2, A). When TDZ concentration increased to 1.0 mg l^{-1} (media I5, I6, I7), the regeneration rates were higher for 2 weeks culture on media I than that for 4 weeks culture on media I. The results revealed that shoot-bud differentiation was promoted when leaf explants were exposed to higher TDZ concentration 1mg l^{-1} for a shorter (two weeks) period of time. When prolonged time of induction period, hindered shoot-bud formation as it inducing bigger callus along with shoot buds (data not shown). Dhaka and Kothari [3] reported that the induction of shoot buds from embryonal cotyledons of sunflower, the formation of callus took place along with shoot-bud formation, hindered further development of shoot-bud. However, when leaf explants of jujube were exposed to lower TDZ concentration 0.5 mg l^{-1}, longer induction period was better to induce shoot-bud formation. And at low TDZ concentration 0.1 mg l^{-1}, regeneration rates were very low no matter how long induction period was on media I. Therefore, the proper TDZ concentration for inducing shoot bud formation from sour jujube leaf explants was 0.5 or 1.0 mg l^{-1}.

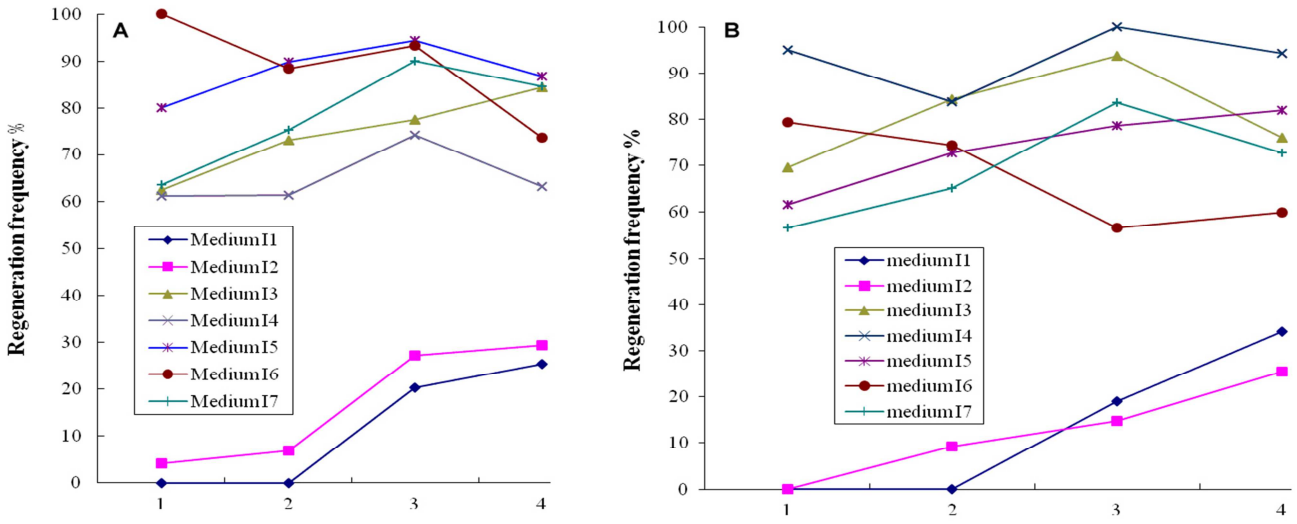

Figure 2. *Effect of duration of Taishan sour jujube leaf explants on induction medium (medium I) on adventitious bud regeneration.*

A: leaf explants cultured for 2 weeks on media I before subculturing on media II; B: leaf explants cultured for 4 weeks on media I before subculturing on media II.

The duration of leaf explants incubation on media I before transferring to media II, affected the process of shoot-bud expression. When leaf explants incubation on media I for 2 weeks, the shoot buds were clearly observed with the naked eye after culturing on media II for 2 weeks (namely 4 weeks from the beginning of leaf explants culture). At this period, the percentage of shoot-bud formation and expression completed more than 50% of final regeneration frequency (data not shown). However, when the incubation on media I for 4 weeks (namely 4 weeks from the beginning of leaf explants culture), no shoot bud formation was observed, only when they were transferred to media II, the shoot buds were observed after 1 week (namely 5 weeks from the beginning of leaf explants culture), the percentage of shoot-bud formation and expression also reached over 50% of final regeneration frequency (data not shown). The results showed that the duration of leaf explants on medium I affected the process of shoot-bud expression, shoot buds production on media II was 1 week earlier comparing 2 weeks with 4 weeks duration on medium I. This further confirmed that medium I was to induce shoot-bud primordium formation and was necessary for adventitious bud initiation, and medium II was to promote shoot-bud growth and elongation and was necessary for adventitious bud elongation.

3.3. Shoot-Bud Regeneration from Leaf Explants Cultured Under Photoperiod or Dark

In the third experiment, the effect of photoperiod culture and the length of dark culture period on shoot bud regeneration was investigated. Shoot buds regeneration succeeded both in photoperiod culture and 1 week or 2 weeks dark culture (Table 3), but all formed buds didn't elongate. Under photoperiod or under 1 week or 2 weeks dark culture, the buds were direct regeneration without along with callus formation (Figure 1, H and I), but regeneration frequency obtained experiencing 1 week or 2 weeks dark culture was lower than that photoperiod culture. This result was similar to previous report that shoot regeneration frequency of barley immature embryos was higher cultured in photoperiod than in dark for 'Lenins' variety [19]. But shoot regeneration frequency was higher cultured in dark than in photoperiod for 'K-3' variety. This showed that the effect of light on shoot regeneration varied with the genotype. Presented here results showed: darkness or photoperiod culture both are effective for adventitious bud regeneration from sour jujube leaf explants, but enough long darkness culture is necessary for shoot bud elongation.

Table 3. *The effect of the length of dark culture period on adventitious bud regeneration of Taishan sour jujube leaf explants after 5 weeks of culture.*

Media II*	Photoperiod (16/8, light/dark)		1 week in dark		2 weeks in dark	
	%	Shoot- bud growth	%	Shoot- bud growth	%	Shoot- bud growth
II1	89.6 ± 3.2 b	Hyperhydric buds	66.3 ± 1.5 b	Hyperhydric buds	69.9 ± 5.4 b	Hyperhydric buds
II2	88.7 ± 3.0 b	Hyperhydric buds	64.0 ± 5.0 b	Hyperhydric buds	69.7 ± 5.7 b	Hyperhydric buds
II3	100 ± 0.0 a	Normal buds	78.8 ± 3.4 a	Normal buds	82.9 ± 7.8 a	Normal buds
II4	85.3 ± 5.4 b	Normal buds	58.3 ± 6.4 b	Normal buds	70.0 ± 5.2 b	Normal buds

*: Leaf explants were transferred to media II after culturing 2 weeks on media I5.
The same letter in each column denotes no significant difference at $p < 0.05$ by LSD test.

The study demonstrated that TDZ promoted adventitious bud differentiation from leaf explants of Taishan sour jujube, while the elimination of TDZ was necessary to promote bud elongation growth. The results supported the previously reports that TDZ promoted adventitious bud differentiation, but the elimination or reduction of TDZ was necessary to promote bud elongation [7, 12].

4. Conclusion

The optimal culture protocol of shoot regeneration from leaf explants of sour jujube was two-step culture, namely, first cultured on WPM containing 1.0 mg l^{-1}TDZ and 0.5 mg l^{-1} IAA for 2 weeks in dark, then transferred and subcultured on WPM containing 0.1 mg l^{-1} IAA and 0.5 mg l^{-1}GA3 for another 2 weeks in dark. The regeneration frequency reached 100%.

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References

[1] Amutha S, Muruganantham M, Ganapathi A (2006) Thidiazuron-induced high-frequency axillary and adventitious shoot regeneration in *vigna radiata* (L) Wilczek. In Vitro Cellular Developmental Biology - Plant 42:26-30.

[2] Brand MH (1993) Agar and ammonium nitrate influence hyperhydricity, tissue nitrate and total nitrogen content of serviceberry *(Amelanchier arborea)* shoots *in vitro.* Plant Cell, Tissue and Organ Culture 35: 203-209.

[3] Dhaka N, Kothari SL (2002) Phenylacetic acid improves bud elongation and in vitro plant regeneration efficiency in *helianthus annuus* L. Plant Cell Reports 21:29-34.

[4] Gao Q, Wang B, Saimaiti T, Xu M, Luo S (2016). Agrobacreium-mediated CBE genetic transformation of Hami *Zizyphus jujube*. Northern Horticulturae, (18):94-98.

[5] Gu XF, Zhang JR (2005) An efficient adventitious shoot regeneration system for Zhanhua winter jujube (*Zizyphus jujuba* Mill.) using leaf explants. Plant Cell Reports 23:775–779.

[6] Huang S, Xie S, Huang C, Zhang Z (2002) Studies on tranquillizing effect of jujuboside. Journal of Dalian university 23:90-92.

[7] Kim MK, Sommer HE, Bongarten BC, Merkle SA (1997) High-frequency induction of adventitious shoots from hypocotyl segments of *liquidambar styraciflua* L. by thidiazuron. Plant Cell Reports 16:536-540.

[8] Liu C, Zhang X, Ma H (1995) Studies of techniques of miniature propagation in wild jujube. Acta Bot Boreal-Occient Sin 15:301-306.

[9] Liu X, Ren Y, Ren X (2004) Study on the relation of tissue structure and drought resistance on *Zizyphus spinosus* (Bunge) Hu. Leaf. J of Henan Vocation-Technical Teachers College 32:45-47.

[10] Li A, Zhang G, Wang X, Li S, Shi D (2000) Preliminary report on biological characteristics and reproduction techniques of medical plant – *Zizyphus jujuba* var. *spinosa* Hu. Inner Mongolia Forestry Science & Technology 2:29-32.

[11] Lloyd GB, McCown BH (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot culture. Combined Proceedings – International Plant Propagator's Society 30:421-427.

[12] Lu CY (1993) The use of thidiazuron in tissue culture. In Vitro Cellular Development Biology-Plant 29:92-96.

[13] Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology Plant 15:473-497.

[14] Nie H, Ye J, Liu K, Zhang Y, Han D (2005) A study of sour jujube as rootstock for jujube. Northern Fruits 3:34-35.

[15] Pan Q (2002) Advances of jujue witches' broom research and control methods. Beijing Agricultural Science 3:4-8.

[16] Peng Z, Zhang H, Cheng S, Guo W (1995) Protective effect of semen *Ziziphi Spinosae* on SOD reduction in mice with endotoxin fever. J of Chinese herb 20:369-370.

[17] Rai VR (2002) Rapid clonal propagation of *Nothapodytes foetida* (Wight) Sleumer – A threatened medicinal tree. In Vitro Cellular Development Biology-Plant 38:347-351.

[18] Reyes-Vera I, Potenza C, Barrow J (2008) Hyperhydricity reversal and clonal propagation of four-wing saltbush (*Atriplex canescens*, Chenopodiaceae) cultivated *in vitro.* Australian Journal of Botany 56: 358–362.

[19] Rikiishi K, Takakazu M, Masahiko M, Kazuyoshi T (2008) Light control of shoot regeneration in callus cultures derived from barley (*Hordeum vulgare* L.) immature embryos. Breeding Science 58:129-135.

[20] Shao X, Wang Z, Zhang J, Feng S, Gao F, Zheng J (2005) Afforestation technology of jujube trees based on wild jujube in Taihang mountain area. J of Mountain Science 23:381-384.

[21] Sun Q, Sun H, Zheng H, Shi Y (2000) Study on the plant regeneration from leaves of sour jujube (*Zizyphus spinosus* Hu.). Journal of Friut Science 17:48-51.

[22] Sun Q, Liu Q, Sun H (2002) Effects of culture conditions on shoot regeneration from leaves of sour jujube (*Zizyphus spinosus* Hu.). Journal of Friut Science 19:24-26.

[23] Sun Q, Sun H., Zhou G (2016). Study of adventitious bud regeneration from stem segments of jujube cultivar Luzao3. Shandong Agricultural Sciences 48 (2): 12-14.

[24] Wang B, Peng D, Liu L, Sun Z, Zhang N, Gao S (2007a) An effficient adventitious shoot regeneration system for ramie (*Boehmeria nivea* Gaud) using thidiazuron. Botanical Studies 48:173-180.

[25] Wang H, Wu Y, An F, Gu P, Zhang Z (2007b) The homology analysis for sequences of 16S rDNA and *tuf g*ene of phytoplasma from jujube witches′ broom and wild jujube witches′ broom. Scientia Agricultura Sinica 40:2200-2205.

[26] Xu M, Dai P, Luo Sh. Gao Q, Ayidingguli H (2014). Studies on adventitious bud regeneration system of *Zizyphus jujuba* Hami Leaves. Journal of Xinjiang Agricultural University 37 (3):185-190.Zheng Y, Qian S, You Z (2006) Advances in study on pharmacological effects of semen *Zizyphus spinosae*. Sichuan Journal of Physiology Science 28:35-37.

[28] Zhou J, Liu M, Hou B (1998) Advances in research on witch-broom disease of Chinese jujube. Journal of Friut Science 15:354-359.