Organogenic Regeneration of an Elite Cultivar of Chinese Jujube (*Zizyphus jujuba Mill*)

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Abstract— *An efficient and relatively simple regeneration system was developed for an elite cultivar of Chinese Jujube, a perennial tree, by culturing young twig segments as explants from 8-15 year old trees. The twig segments were disinfected by submerging them in 1% sodium hypochlorite (NaOCl) for 15 min with 3 min vacuum. Calli developed from both ends of the twig segments on half-strength MS medium supplemented with sucrose and BA or BA and NAA in combination. The frequency of shoot formation from calli was higher than 80% when the explants were placed on the half - strength MS medium supplemented with BA (2.581 µM) and NAA (2.685 µM). Roots were produced from adventitious buds for 90% of the regenerated shoots when they were placed on the MS medium supplemented with 4.920 µM IBA and 5.708 µM IAA. After transplanting to soil, 82% of the regenerated seedlings survived when they were covered with glass containers to maintain humidity. The results suggest that Chinese jujube can be reproduced and multiplied using organogenesis with the appropriate explant and culture medium.*

Keywords— explants, regeneration, vacuum.

I. INTRODUCTION

Chinese jujube (*Zizyphus jujuba* Mill) is a fruit tree grown in vast areas of China because of its wide adaptation to various climates and high degree of tolerance to barren soil (Mengjun, 2003). The species are rich in sugars and vitamins, and this fact has made *Ziziphus* species important fruit trees for many centuries, including China, India, Persia, Armenia, the Mediterranean and in the dry regions of the southwestern US (Sweet, 1985; Reich, 1991; Gilman and Watson, 1994; Hache and Xu, 1995; Outlaw et al., 2002). The fruit has been used medicinally for millennia by many cultures. The total jujube growing area in China is about 66.7 million ha with annual fruit production of 780,000 tons (Qu and Wang, 1993). However, jujube production is limited by damages caused by the peach fruit borer (*Carpasina niponesis* Wds) and the witch-broom disease incited by Mycoplasma - like organisms. Evidence indicates that approximately 7,500,000 kg of jujube are lost annually in a production base in the Taihang Mountain area in China owing to infestation by the pest (Liu et al., 1997).

The fruit is rich in vitamin C (500 mg/g), flavone, and 18 amino acids (Chen et al., 1996; Romero Rodriguez et al., 1992; Arndt et al., 2001). The commercial importance of jujube has increased in recent years because of the recognition of the nutritional value of the fruit. However, some nutrients are degraded during the drying and processing of the fruit for market. Consequently, methodology needs to be developed to increase shelf life of fresh jujube to bypass the drying and processing required for marketing.

With the advent of biotechnology, incorporation of desirable genes from alien species using gene transfer techniques has resulted in the development of Roundup - Ready soybean, Bt corn and Bt cotton (Engel et al., 2002). Likewise, transferring insect and disease resistance genes as well as genes that can prolong fruit shelf life into superior cultivars of jujube could increase quality and expand production of this fruit crop.

Most jujube cultivars produce fruit without cross-pollination, but seeds from such self-pollination are usually not viable (Reich, 1991). Most Chinese cultivars are vegetatively propagated by grafting or budding onto a thorny rootstalk which produces many suckers from the roots. There is evidence that jujube cultivars will root on hard or soft wood cuttings. However, successes have been limited to date with this process of plant reproduction. Micropropagation has been practiced in the past to multiply the Chinese jujube in large quantities that is more efficient than conventional multiplication by soft cutting (Cheong and Kim, 1984; Cheong et al., 1987; Mitrofanova et al., 1994; Gu et al., 2008; Soliman and Ghada Hegazi, 2013; Shi et al., 2015). To date, successful plant regeneration has been reported in *Z. jujuba* from stem (Mathur et al., 1995)

and leaf (Gu et al., 2005). However, the regeneration frequency was low. Thus an alternative approach must be developed to improve efficiency of regeneration so that it could be applied for large - scale commercial production.

The purpose of this study was to test the feasibility of using young twigs from mature trees growing in the field as explants for developing a simple and reliable regeneration system for Chinese jujube.

II. MATERIALS AND METHOD

A. Plant material

Young actively growing twigs produced between May 10 and July 20 were excised from 8-15 year old trees of "Jun", a high quality cultivar of Chinese jujube. After the leaves and shoot tips were removed, twigs with one bud were submerged in tap - water containing a few drops of the neutral detergent (Sun Spray) for 5 min, rinsed with running tap water for 10 min, and re-submerged in 70% (v/v) ethanol for 30 s. Then three immersion regiments were tested for efficacy of sterilization and included: 0.1% mercuric chloride (w/v) for 15 min, 1% sodium hypochlorite (w/v) for 15 min, and 1% sodium hypochlorite (w/v) for 15 min with the first 3 min under vacuum. Then twigs were rinsed three times with the sterile distilled water at 2 min each, and dried with sterilized paper towels. Both ends of the twigs were given a fresh cut, and the remaining section was cut (at 45E angle) into a 1 cm segments with one node. Fifteen segments were used in each replicate for each treatment and 10 replicates were recorded.

B. Bud differentiation and shoot production.

Fifteen twig segments were placed in each petri dish (90 × 18 mm) containing 30 ml of half - strength of mineral salts of MS medium (Murashige and Skoog, 1962) supplemented with 15 g of sucrose and various concentrations of BA (0.26, 1.29, 2.58, 3.87, and 5.16 μM) alone or in combinations with either 2.68 μM NAA or 1.156 μM IAA. The liquid medium was adjusted with 0.1 KOH to pH 5.8, then add 7g Difco agar before autoclaving (121 ℃ at 103.5 kPa) for 15 min. Each Petri dish is considered as one replicate. Each treatment was replicated three times, and the experiment was repeated three times over three years.

Petri dishes containing twigs were kept in a growth room at 27±2℃ under a 16 h photoperiod supplemented by cool - white fluorescent lights at an intensity of 60 μmol m-2s-1. The number of developing shoot longer than 1 mm counted after 4 weeks.

C. Shoot elongation, rooting and acclimatization.

Shoot buds were transferred to 150 ml Erlenmeyer flasks containing 50 ml half - strength MS with 15 g l-1 sucrose and 7 g l-1 Difco agar but without growth regulators. The length of shoots was measured 6 weeks later. Shoots longer than 2 cm were excised and transferred to flasks containing 50 ml of fresh medium and supplemented with either (1) 5.708 μM IAA, (2) 4.920 μM IBA, or (3) 5.708μM IAA + 4.920 μM IBA to promote rooting. Each treatment included 15 shoots and was replicated twice.

Shoots were maintained in a growth room at 25±2℃ under a 16 h photoperiod with cool - white lights at 90 μmol m-2s-1. After another 4 weeks, plantlets were transferred to plastic cups containing sterilized sand covered with a glass cup to maintain humidity. Plantlets were acclimatized at 25℃ in sunlight filtered with a shade cloth for two more weeks using shading, and finally transferred to 5 cm pots containing soil and peat mix.

Growth and development were monitored at several stages during in vitro regeneration. Stem segments were examined for contamination and general appearance 10 days in culture. Shoot buds were counted on the callus formed at the cut stem ends after 4 weeks on shoot initiation medium. Numbers and lengths of roots were measured at 4 weeks following transfer to rooting medium. The number of developing shoot buds longer than 1 mm was counted after 4 weeks.

Data including numbers of contaminated twig segments and segments showing visible necrosis for each of the three treatments were recorded for each replicate. Then analysis of variance for percent of healthy segments was made and differences were compared using least significant difference (Steel and Torrie, 1960).

III. RESULTS AND DISCUSSION

A. Effectiveness of sterilization.

Treating explants to 1% sodium hypochlorite (w/v) with the vacuum treatment was the most effective surface - sterilization method for reducing contamination (Table 1). Surface - sterilization with 0.1% mercuric chloride resulted in necrosis and or browning of stem segments. High rate of contamination occurred when segments were immersed in 1% sodium hypochlorite, unless a vacuum was applied during the first 3 min of the 15 min sterilization period (Table 1).

TABLE 1
EFFECTS OF VARIOUS STERILIZATION METHODS FOR TWIG SEGMENTS OF CHINESE JUJUBE

Methods	Total no. segments treated	No. segments contaminated	No. segments showing necrosis	Effective sterilization rate (%)
0.1% $HgCl_2$ for 15 min	150	5	120	16.7
1% NaOCl for 15 min	150	127	2	14.0
1% NaOCl for 15 min with 3 min vacuum	150	6	9	90.0

B. Bud differentiation and shoot production

Five days following culture, calli began to form along both ends of stem segments. Shoot buds differentiated on light yellow, but not white, friable callus developing on the cut ends of the stem segments. Bud initiation began 2 weeks after culture (Fig.1). Concentrations of growth regulators in combination appear to have significant impact on bud and shoot development (Fig. 2). Additions of BA at concentrations in the range of 1.29-3.87 μM in combination with NAA (2.68 μM) or IAA (1.14 μM) were most effective for inducing buds. The highest frequency of bud formed on media supplemented with 2.58 μM BA and 2.68 μM NAA, or 1.29 μM BA with a 1.14 μM IAA, which showed 85% and 80% success rates, respectively, and were significantly superior than other treatments ($P < 0.01$). In the absence of auxins, bud differentiation rate was very low (Fig. 2A).

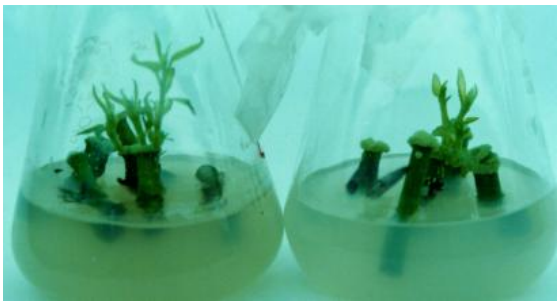

FIGURE 1. BUDS INITIATION AFTER CULTURING 2 WEEKS

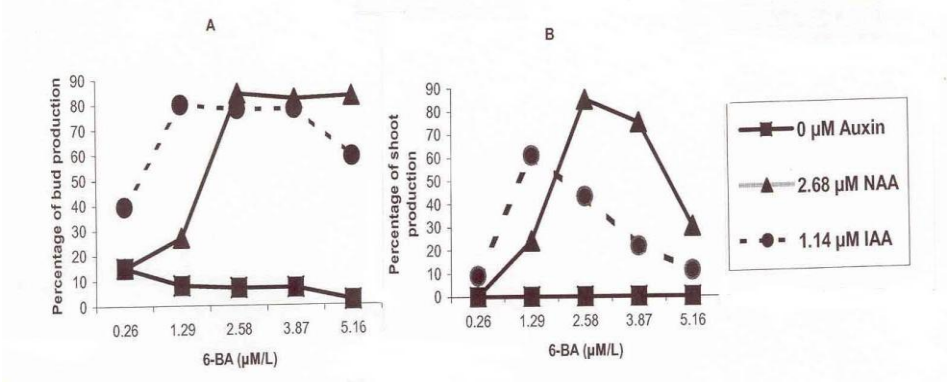

FIGURE 2. EFFECTS OF COMBINED GROWTH REGULATORS ON BUD AND SHOOT DEVELOPMENT OF JUJUBE

The positions of segments on the young twigs had no observable effect on adventitious bud formation or shoot production (data not shown). Buds could develop from the callus formed on either or both ends of the stem segment. A maximum of five buds formed on one segment. But usually only one bud developed into a shoot. Excision of the developing shoot would result in another bud developing into a shoot. Occasionally, two buds were formed at the same time.

C. **Shoot elongation, rooting and acclimatization**.

Shoot elongation was dependent on the concentration of BA and NAA in the medium. The best results for shoot production were observed on the medium supplemented with 2.58 μM BA and 2.68 μM NAA.

The rooting experiment indicated that IBA was more effective than IAA in stimulating root formation from shoots cultured in vitro (Fig. 3A, B). A combination of growth regulators was more effective than IBA or IAA used alone as shown by the root numbers per plantlet. In addition, a combination of growth regulators also improved shoot elongation (Fig. 3C). These results were in agreement with those for cultivar "Minshandazao" reported by Zhang et al. (1995).

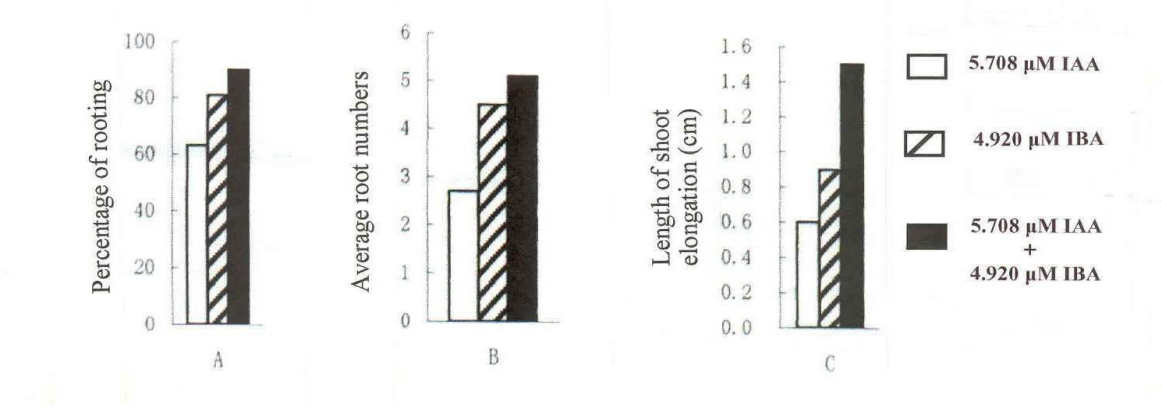

FIGURE 3. IAA, IBA AND THEIR COMBINATION INFLUENCE ROOTING RATIO, ROOT NUMBERS AND SHOOT ELONGATION OF JUJUBE

Acclimatization is an important step for transplanting of in vitro regenerated plantlets to soil in pots. With the use of glass cups to cover plantlets and maintain humidity, the survival rate of plantlets transferred to soil reached 82% in this study (Fig. 4).

FIGURE 4. IN VITRO REGENERATED PLANTLETS TRANSFERRED TO SOIL IN POTS

IV. CONCLUSION

The current study describes methodology which provides an 80% efficiency of plantlet regeneration and survival for Chinese jujube. Young twigs appeared to be a much better source of explants than the leaf discs reported before (Chen et al., 1996). BA promoted bud differentiation, and the additions of IBA and IAA were essential for root induction and shoot elongation. This was the first report using young twigs as explants for Chinese jujube fruit tress, and the improved culture system is relatively simple but effective in producing high frequencies of calli that in turn regenerate well.

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