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Morphogenesis *in Vitro* of *Fritillaria* spp.

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ABSTRACT

The genus *Fritillaria* includes 100 species of bulbous plants and is found throughout the temperate region of the Northern Hemisphere. *Fritillaria* species are often used as ornamental plants, but various species have also been used in traditional Chinese, Japanese and Turkish medicine. Many species from the genus *Fritillaria* are endangered, rarely found in the wild and protected by law. Micropropagation techniques have great importance for germplasm conservation and commercial multiplication of fritillaries. Successful propagation methods have been developed for the following *Fritillaria* species: *F. anhuiensis*, *F. alburyana*, *F. camtschatcensis*, *F. cirrhosa*, *F. hupehensis*, *F. imperialis*, *F. meleagris*, *F. pallidiflora*, *F. przewalskii*, *F. roylei Hook*, *F. sinica*, *F. sichuanica*, *F. thunbergii*, *F. taipaiensis*, *F. unibracteata*, *F. ussuriensis* and *F. whitallii.* This paper summarises the various techniques of *in vitro* morphogenesis induction and rapid propagation of fritillaries, as well as successful acclimatisation. The most potent explant types for the induction of morphogenesis *in vitro* are bulbs, bulb scales, inflorescence parts and immature or mature zygotic embryos. Whole plant regeneration of fritillaries has been achieved by bulblet production, as well as by direct or indirect somatic embryogenesis. The influence of different media compositions, hormone concentrations and temperature requirements for the induction of morphogenesis and overcoming of dormancy are discussed. This review also describes major secondary metabolites in *Fritillaria* (alkaloids and non-alkaloid constituents), their nature and perspective for production by methods of *in vitro* culture which can be used in the pharmaceutical industry.

Keywords: alkaloids, bulblets, fritillary, somatic embryogenesis

Abbreviations: B5, medium according to Gamborg *et al*. (1968); **BA**, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **EB**, 24-epibrassidinolide; **ER**, medium according to Erickson *et al*. (1965); **Fa**, medium according to Fast *et al*. (1981); **IAA**, indole-3-acetic acid; **2iP**, 2-isopentenyl adenine; **KIN**, kinetin; **LS**, medium according to Linsmaier and Skoog (1965); **MN**, medium according to Wu and Tang (1992b); **MS**, medium according to Murashige and Skoog (1962); **NAA**, α-naphthaleneacetic acid; **N6**, medium according to Chu *et al*. (1975); **SOD**, superoxide dismutase; **TDZ**, thidiazuron; **Z**, zeatin riboside

CONTENTS

INTRODUCTION

The genus *Fritillaria* belongs to the family *Liliaceae* and comprises about 100 species with medical value; because of the beauty of their flowers, these plants also have an ornamental value. According to several authors, the number of species may be as high as 160, because some species may be separated according to anatomical (Corneanu and Popescu 1981; Naohiro and Kenji 2006; Zhou *et al*. 2007) and genetic studies (Tsoi *et al*. 2003; Li *et al.* 2009). The natural habitat of fritillaries is the temperate region of the Northern Hemisphere, and the highest number of species is found in Turkey, China, California and Greece. Recent data indicate that its primary evolutionary centre is Iran (Kamari and Phitos 2006).

Ornamental species of the genus *Fritillaria* are very

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Invited Review

popular in gardens because of their attractive bell- or cup-shaped flowers or variable colours, often with a checkered pattern. Some species like *F. imperialis*, the Persian fritillary, have been known for a very long time in Eastern Europe and Iran, and it has been claimed that a significant harvest was performed in the 16th century in an effort to cultivate it (Alp *et al*. 2009).

The very high content of alkaloids (valuable secondary metabolites) in the bulbs of many fritillaries has led to their use in traditional medicine in China, Turkey and Japan for many centuries. Its name in traditional Chinese medicine is Beimu, which refers to the appearance of the bulb of fritillary plants which is similar to that of the cowry shell (bei) of which the plant is the source (mu=mother). Also, many modern prescriptions contain *Fritillaria* components (Dharmananda 2010). These remedies are used today for lung disorders, cough and blood pressure modulation, and possess anti-tussive, anti-asthmatic and expectorant properties as well (Li *et al*. 2006). Besides alkaloids, the starch content in *Fritillaria* species is very high and it might be considered as a new starch source in the food industry (Wang *et al*. 2005a, 2005b, 2005c; Li *et al*. 2011).

Many *Fritillaria* species are endemic, while others are very rarely found in the wild and are often protected by law in their native countries (Kizil *et al*. 2008; Chauhan *et al*. 2011; Badfar-Chaleshtori *et al*. 2012; Mancuso *et al*. 2012). In recent years, many studies have been conducted for improving multiplication and returning endangered plants to their natural environment (Jevremović *et al.* 2006). Numerous *Fritillaria* species are endangered bulbous plants, and therefore micropropagation has great importance for germplasm conservation and commercial production. Recently, cryopreservation as a method for long term conservation of plant material in liquid nitrogen (-196°C) has applied for *Fritillaria* sp. The shoot tips of *F. anhuiensis* were successfully cryopreserved by vitrification method (Zhu *at al*. 2011).

Fritillaries demonstrate very low vegetative propagation in nature, which is a problem for mass production and cultivation (Carosso *et al*. 2011). Rapid expansion of the pharmaceutical industry has increased the demand for medicinal plants, leading to over-exploitation. In recent decades, plant tissue culture has become an alternative method for the propagation of many rare plant species which are threatened in the wild, but also for the increase in plant propagation for pharmaceutical purposes without destroying these plants in their natural habitat (Nalawade *et al*. 2003). *In vitro* techniques have been shown to be a suitable solution for the multiplication of many *Fritillaria* species (Hao *et al.* 1982; Kukulezanka *et al*. 1989; Sun and Wang 1991; Paek *et al.* 1996; Witomska and Lukaszewska 1997; Otani and Shimada 1997; Gao *et al*. 1999; Joshi *et al*. 2007).

Plant regeneration of fritillaries has been reported by organogenesis (bulb production), somatic embryogenesis and androgenesis. Explant choice, media composition and other factors, such as the effect of low temperatures on breaking dormancy are discussed in detail. In this review, we summarise all the aspects of *in vitro* morphogenesis of most *Fritillaria* species that have been propagated by culture *in vitro* up to date.

CLASSICAL PROPAGATION

Fritillaria species are bulbous plants which are propagated in nature via seeds and vegetatively by daughter bulbs. Their bulbs are composed of a flower bud, scales, roots, basal stem and tunic. The bulbs accumulate nutrition and stay dormant during the winter months. Most ornamentally and medically valuable fritillaries bloom in April, and the best bulb yield is achieved with planting in September. Dormancy, which is manifested during the *Fritillaria* life cycle, is an essential condition for their normal development. For most fritillaries, the period of dormancy starts in the beginning of the summer, when the aerial part dries up. The low temperature during the winter breaks dormancy and allows a vegetative period and flowering in the spring. Species from the genus *Fritillaria* are usually geophytes which spend about 80-90 days of their life cycle above the ground and 270-280 days beneath the ground (Zhu *et al*. 1980; Sun and Wang 1991). In this way, propagation is very low since one mother bulb can produce only 2-3 bulblets, depending on the environmental conditions and vegetative techniques (Ulug *et al*. 2010).

Sexual propagation by seeds is slower than vegetative propagation and takes much more time for plant growth. The seedlings are weak and it can take 4-6 years for growing from the initial material to maturity (Paek *et al*. 1996; Witomska and Lukaszewska 1997; Gao *et al*. 1999).

PROPAGATION *IN VITRO*

Research on *Fritillaria* propagation by tissue culture *in vitro* started with medically valuable species of the genus, such as *F. thunbergii* (Sun *et al*. 1977), *F. pallidiflora* (Hao *et al*. 1982) and *F. ussuriensis* (Zhao *et al*. 1983). After the first results, many reports were published about the successful induction of morphogenesis *in vitro* by bulb culture or somatic embryo production of other fritillaries (**Tables 1, 2**). Success depends on the developmental stage of the plant, explant choice, composition of the basal medium, the combination of hormones and the *in vitro* technique used. Plantlets are regenerated by bulb production, somatic embryogenesis and androgenesis.

Bulb production

Plant regeneration of fritillaries by organogenesis, i.e. bulb formation, has so far been reported for 17 species. Bulb production can be induced from different initial explant types, such as bulbs, bulb scales, stems, inflorescence and flower parts (**Table 1**).

1. Bulb and bulb scale culture

Bulb tissues may be cultured as the whole bulb (Kukulezanka *et al*. 1989; Gao *et al*. 1999), as a vertical scale (Paek and Murthy 2002), or as a transverse scale section (Joshi *et al*. 2007). Sterilisation of fritillary bulbs is usually simple; the standard procedure can be applied with some modification (e.g., 15-20 min in sodium hypochlorite, 70% alcohol, 0.1% mercuric chloride, 2% chloramine and sterile distilled water). To reduce possible contamination, which is often associated with bulbs as initial explants, antibiotics such as kanamycin, vancomycin and cefotaxime can be added (Paek *et al*. 1996; Seon *et al*. 1999) or advanced techniques can be used for the sterilisation of materials taken from nature (Witomska *et al*. 1998).

Regeneration from bulbs and bulb scales of fritillaries depends on the species, mineral composition of medium, composition and concentration of hormones, concentration of sugar, light regime, age and size of the mother bulb. Early reports from 1982 showed that harvested bulbs of *F. pallidiflora*, cut into small pieces, and cultured on media with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) (1.0 and 0.1 mg/l, respectively) produced calli. Callus induction reached 35% after 30 days of culture on MS medium. Addition of B5 (Gamborg *et al*. 1968) medium had no effect on callus production (Hao *et al.* 1982).

Gao *et al.* (1999) examined the effect of various culture media on the growth rate and yield of bulbs of *F. unibracteata*. MS medium was the most suitable. After 40 days of culture, bulbs were harvested and tested on different concentration of hormones indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA). BA (1.0-2.0 mg/l) and IAA (0.5-1.0 mg/l) were favourable for bulb culture. The best combination for a high growth rate and yield of bulbs was IAA and BA at 1.0 mg/l each. These results suggest that the growth rate increased with culture age; the highest growth rate was achieved after 50-

Table 1 List of *Fritillaria* species regenerated by bulb culture.

Species	Explant	Medium (plant regulators in mg/l)	Reference
F. alburyana	bulb scale, immature embryo	MS + NAA 0.25 + BA 4.0; N6 + 2,4-D 2.0	Özcan *et al.* 2007
F. anhuiensis	bulb	MS + NAA 2.0 + KIN 2.0	Xue *et al.* 2008
F. camtschatcensis	bulb scale, leaf	LS + NAA 0.1 or picloram 0.1	Otani and Shimada 1997
	bulb scale	MS + NAA 0.1 + EB 0.1	Okawa and Kitajima 1998
	bulb scale	MS + NAA 1.0 + KIN 0.1 + EB 0.01	Okawa *et al*. 1999
	bulb	MS + NAA 0.1 + KIN 0.1	Okawa and Nishino 2000
F. cirrhosa	bulb scale	MS + NAA 1.0 + BA 2.0	Wang *et al*. 2002
	bulb scale	MS + NAA 0.2 + BA 2.0	Wang *et al*. 2010a
F. hupehensis	bulb scale	MS + NAA 4.0 + BA 0.5	Chen *et al*. 2000; Yang *et al*. 2001
F. imperialis	bulb scale, shoot part, flower part	MS + NAA 0.5 + BA 1.0	Witomska and Lukaszewska 1997, 1998; Lukaszewska *et al*. 1998
	petal	MS+ NAA 0.6 + IAA 0.4 + BA 1.0	Mohammadi-Dehcheshmeh *et al*. 2008
F. meleagris	seed, bulb	MS or FA + NAA 1.0 + BA 0.5-2.0	Kukulezanka *et al*. 1989
	zygotic embryo	MS + 2,4-D or TDZ 1.0	Subotić *et al.* 2004
F. pallidiflora	bulb scale	MS + 2,4-D 1.0 + KIN 0.1; MS + IAA 1.0 + NAA 0.2 + KIN 0.1	Hao *et al*. 1982
	bulb	MS + 2,4-D + KIN; MS + NAA 1.0 + KIN 0.1	Wang *et al*. 1987
	bulb	MS + IAA 1.0 + NAA 0.2 + KIN 0.1	Sun and Wang 1991
F. przewalskii	bulb	MS (or MS/2) + NAA 2.0 + 2,4-D 2.0 + BA 0.5 + IBA 0.5 + KIN 0.2	Wang *et al*. 2009
F. roylei Hook	bulb scale	MS + NAA 1.0 + KIN 1.1	Joshi *et al*. 2007
F. sichuanica	bulb scale	MS + 2,4-D 1.0 + KIN 0.1; MS + NAA 0.2 + IAA 0.1 + KIN 0.1	Qiao *et al*. 1986
F. sinica	young stem	MS + NAA 1.0 + BA 0.5	Hao *et al*. 1995
F. taipaiensis	bulb scale	MS + NAA 1.0 + BA 3.0	Liu *et al*. 1996
F. thunbergii	young leaf	MS + NAA 2.0 + KIN 1.0	Sun *et al*. 1977
	bulb scale, flower stem	MS + NAA 0.3 + KIN 1.0	Paek *et al.* 1996; Seon *et al.* 1999
	bulb scale	MS + NAA 0.1 + KIN 1.0	Paek and Murthy 2002
	bulb	MS + NAA 1.0 + Z 2.0	Yuan *et al*. 2005
F. unibractetata	bulb explant	MS + IAA 1.0 + BA 1.0	Gao *et al*. 1999
F. ussuriensis	bulb scale	MS + NAA 0.5 + KIN 1.0	Zhao *et al.* 1983
		MS + NAA 0.5 + KIN 1.0	Sun and Wang 1991
	root, stem, leaf	MS + MS + IBA 1.0 + Z 1.0; MS + IAA 0.5 + BA 2.0; MS + NAA 2.0 + KIN 0.5	Wu and Tang 1992a
	bulb	MS + NAA 4.0; MS + NAA 2.0 + BA 0.5; MS + 2,4-D 2.0 + BA 0.5	Sun *et al*. 2008
F. whittallii	bulb scale, immature embryo	MS + NAA 0.25 + BA 4.0; N6 + 2,4-D 2.0	Özcan *et al*. 2007

BA, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **EB**, 24-epibrassidinolide; **Fa**, medium according to Fast *et al*. (1981); **IAA**, indole-3-acetic acid; **KIN**, kinetin; **LS**, medium according to Linsmaier and Skoog, (1965); **MS**, medium according to Murashige and Skoog (1962); **NAA**, α-naphthaleneacetic acid; **N6**, medium according to Chu *et al*. (1975); **TDZ**, thidiazuron; **Z**, zeatin riboside

Table 2 List of *Fritillaria* sp. regenerated by somatic embryogenesis.

Species	Explant	Medium (plant regulators in mg/l)	Reference
F. alburyana	zygotic embryo	MS + NAA 0.25 + BA 4.0; N6 + 2,4-D 2.0	Özcan *et al.* 2007
F. hupehensis	bulb scale; stem base	MS + NAA 4.0 + BA 0.5	Shiau *et al*. 2000
F. imperialis	petal	B5 + IAA 0.4 + NAA 0.6 + BA 0.1	Mohammadi-Dehcheshmeh *et al*. 2007
F. meleagris	zygotic embryo	MS + 2,4-D 1.0 or TDZ 1.0	Nikolić *et al*. 2006: Petrić *et al*. 2011
	leaf base	MS + 2,4-D or KIN	Subotić *et al*. 2010
	in vitro bulb scale	MS + 2,4-D 0-10 or TDZ 0-2.0	Petrić *et al*. 2011
F. palidiflora	bulb	MS + IAA 0.1 + KIN 0.5	Wang *et al.* 1989
	bulb	MS + 2,4-D, KIN	Xu and Zhu 1998
F. sinica	young stem	MS + NAA + 2,4-D + BA + KIN	Hao *et al*. 1995
F. thunbergii	bulb	MS + 2,4-D 1.0 + KIN 2.0	Yuan *et al*. 2005
F. ussuriensis	bulb	MN + 2,4-D 2.5 + KIN 1.0; N6 + KIN 1.0	Wu and Tang 1992b
	bulb	MS + NAA + KIN + BA	Xu and Zhu 1998
	root tip	ER + IAA 2.0 + 2,4-D 0.1 + BA 1.0; ER + IAA 0.1 + BA 1.0	Feng *et al.* 2009

B5, medium according to Gamborg *et al.* (1968); **BA**, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **ER**, medium according to Erickson *et al.* (1965); **IAA**, indole-3-acetic acid; **KIN**, kinetin; **MN**, medium according to Wu and Tang (1992a); **MS**, medium according to Murashige and Skoog (1962); **NAA**, α-naphthaleneacetic acid; **N6**, medium according to Chu *et al*. (1975); **TDZ**, thidiazuron

60 days of culture. The optimum time for harvesting bulbs was after 50 days of culture. The authors claimed that with their protocol, the growth rate of bulbs increased 30-50 times compared to natural conditions and it also allowed subculturing of bulbs for a long period of time (Gao *et al*. 1999).

Bulb formation of *F. camtschatcensis* was achieved on LS medium (Linsmaier and Skoog 1965). Bulbs were cut into halves and cultured on LS medium with NAA (0.1-5.0 mg/l), picloram (0.1-1.0 mg/l) and/or BA (1.0 mg/l). After 40 days of culture, explants produced adventitious bulblets. Low concentrations of NAA and picloram had a strong effect on bulblet formation, while BA had no great effect. It was noted that many explants (75%) formed bulblets on hormone-free medium, but the greatest rate (90%) and number of bulblets (five bulblets per explant) was obtained on medium supplemented with NAA (0.1-0.5 mg/l) or picloram (0.1-1.0 mg/l). Different concentrations of sucrose (3, 6 and 9%) implemented in LS medium were also tested. The highest growth rate (three times higher than the initial rate) was achieved on LS medium with 3% sucrose with or without NAA (the applied concentration was 0.1 mg/l for each sucrose concentration). A higher concentration of sucrose led to a small increase in fresh weight. The bulblets produced leaves and roots on LS medium without hormones after 4 months at 20°C (Otani and Shimada 1997).

Bulbs of *F. imperialis*, collected from nature, were cut into eight segments and cultured on MS medium with NAA and BA (0.5 and 1.0 mg/l, respectively). Regeneration was evaluated after 1, 2 and 3 months in darkness but also in the light. One-month old explants formed calli, only 4% regenerated bulblets and regeneration did not increase after 2 or 3 months. There was no regeneration in the dark (Witomska and Lukaszewska 1997).

Paek and Murthy (2002) showed bulblet regeneration of *F. thunbergii* from bulb scale sections at different concentrations of the cytokinins BA, KIN or 2-isopentenyl adenine (2iP) with NAA (0.1 mg/l) in MS medium supplemented with 5% sucrose. The effect of three types of culture (solid, agitated liquid and static liquid culture) was also examined. Bulbs were stored at low temperature to improve regeneration capacity. It was found that KIN (1.0 mg/l) was the most suitable for new bulblet formation after 12 weeks, with *ca*. 14 bulblets/explant. The results revealed that solid and agitated liquid culture medium proved to be the most favourable when the fresh weight of explants together with that of newly formed bulblets was measured. The effects of light–dark (continuous dark and 16-h photoperiod) and different temperature regimes (20, 25 and 30°C) on bulblet regeneration of *F. thunbergii* were also investigated. The highest number of bulblets (12) formed at 25°C under a 16-h photoperiod as compared to nine bulblets at a similar temperature but in constant darkness. With an increase in the sugar concentration, the number, diameter and fresh weight of bulblets increased (Seon *et al*. 1999). The greatest number of bulblets was obtained on medium supplemented with 5% sucrose while the greatest bulblet size was recorded on medium supplemented with 7% sucrose.

Bulb scale explants were efficient as reported for early experiments with *F. ussuriensis* (Zhao *et al*. 1983). In this study, liquid MS medium was used, which was shown to be better than solid medium for bulb differentiation, bulb growth and also root and shoot growth. NAA (0.5–1.0 mg/l) was shown to be the best combination for the induction of calli, which were induced after 3-4 weeks, while NAA and KIN (0.5 and 1.0 mg/l, respectively) were best for bulblet induction when regeneration induction was 65–80%.

Regeneration of *F. meleagris* by bulb production was reported for the first time by Kukulezanka *et al*. (1989). Satisfactory results were achieved on MS and Fa media (Fast 1981) with BA (0.5-2.0 mg/l) or NAA and BA (1.0 mg/l each). Medium supplemented with NAA and BA (1.0 and 2.0, respectively) was optimal for producing new bulblets (7 new bulblets/explant).

Different sections of the same mother bulb might be very important for fritillary bulb production as reported for high mountain *Fritillaria roylei* from the Himalaya (Joshi *et al*. 2007). Surface-sterilised bulb scale explants were cut transversely into two pieces and cultured (one piece from the basal region and another from the distal region). Both types of explants were cultured on MS medium supplemented with NAA, BA or KIN. The number of explants producing bulblets and the number of newly formed bulblets/explant were counted after 8 weeks. Basal scale sections showed significantly better regeneration of explants and a higher number of new bulblets than distal sections. The best results for both observed parameters were achieved on MS media supplemented with NAA and KIN (0.1 and 1.1 mg/l, respectively).

Bulb scales are the most efficient explant type in the case of *F. hupehensis* (Shiau *et al*. 2000). Explants were cultured on MS medium with BA 0.5 mg/l, and after 60 days, 54.7% bulb induction was achieved. The addition of NAA (0.25-4.0 mg/l) increased bulb formation from 62% to 78%. The highest concentration of NAA (4.0 mg/l) could promote further rooting ability of bulbs and 65% of bulblets were rooted (Chen *et al*. 2000).

BA or KIN as cytokinins and mainly NAA as auxins are present in many protocols for successful bulb induction and the multiplication of many fritillaries (**Table 1**). In addition, zeatin (Z) was efficient in root and stem culture of *F. ussuriensis* and also in bulb culture of *F. thunbergii* (Wu and Tang 1992a; Yuan *et al*. 2005). The positive effect of 24-epibrassinolide (EB) on bulblet formation was reported for *F. camtschatcensis* (Okawa and Kitajima 1998). The optimum conditions for bulblet formation were MS medium supplemented with NAA and EB at a concentration 0.1 mg/l each when morphogenesis induction was performed in the dark.

2. Stem and inflorescence parts culture

In addition to bulbs and bulb scales as initial explants, other plant parts have been used for successful morphogenesis induction of fritillaries. This is recommended when the use of bulb scale pieces can result in the destruction of endangered mother plants. Other types of explants are therefore often necessary (Lukaszewska *et al.* 1997, 1998; Mohammadi-Dehcheshmeh *et al*. 2007). Other types of explants may also have a great ability to regenerate (**Table 1**). Other organs which could be used for the micropropagation of fritillaries include leaves (Sun *et al*. 1977; Wu and Tang 1992a; Paek *et al*. 1996; Otani and Shimada 1997; Witomska and Lukaszewska 1997; Subotić *et al*. 2010), stems (Hao *et al.* 1995; Paek *et al*. 1996; Witomska and Lukaszewska 1997; Shiau *et al*. 2000; Paek and Murthy 2002), inflorescence stem segments (Seon *et al.* 1999), petals (Mohammadi-Dehcheshmeh *et al*. 2008), anthers (Du *et al*. 1986) and seeds (Du and Hou 1985; Kukulezanka *et al.* 1989).

Early reports on *F. thunbergii* showed that bulblet formation was significantly increased in node-bud or stem segment culture compared to scale segments which exhibited a high level of contamination (Paek *et al*. 1994, 1996). Inflorescence stem segments of newly formed bulbs from mother bulbs of *F. thunbergii* which were stored for 2-6 weeks in a moist environment at 10°C were shown to be a good explant source (Yu *et al*. 1994; Seon *et al*. 1999). Explants were subcultured several times on MS medium with NAA and KIN (0.3 and 1.0 mg/l, respectively) with the addition of antibiotics to reduce contamination. During cold storage, stem segments were cultured on media supplemented with various concentrations of BA or KIN (1.0-5.0 mg/l). The number of bulblets/explant (26.6 and 20.0, respectively) formed from stem culture on medium containing KIN (1.0 or 3.0 mg/l) was higher than the number of bulblets formed from bulb scales on the same medium (Seon *et al*. 1999). The formation of bulblets in stem culture of *F. thunbergii* depended on the physiological age of the stem. Young stems (smaller than 10 cm) had better regeneration ability than older ones. The optimum level of KIN for bulblet production from old stems was 1.0 mg/l with 5-7% sucrose. The most effective explant for micropropagation was stem tissue excised from plants less than 3 cm in height, with a 20-fold multiplication rate (Paek *et al*. 1996).

Leaves which emerged from bulb scales were used as explants for tissue culture of *F. camtschatcensis* (Otani and Shimada 1997). Leaf explants were cultured on LS medium containing picloram (0.1 or 1.0 mg/l) and adventitious bulblets were formed. The regeneration frequency was much lower (12.5-26.1%) compared to bulb scales cultured with the same medium composition (86-97%).

A very high morphogenetic response of stem explants was also obtained for *F. hupehensis* (Shiau *et al*. 2000). Stem base segments were cultured on MS medium supplemented with BA (0.5 mg/l) and a greater number of bulblets/explant was achieved than with bulb scales. The addition of NAA (0.25-4.0 mg/l) increased the formation of bulblets to 68-72%.

Upper leafy shoot parts of ornamental *F. imperialis* were used as the starting material and proved to be very effective explants, much better than bulb scales. Shoot parts were cultured in MS medium with the addition of NAA and BA (0.5 and 1.0 mg/l) under light or dark conditions (Witomska and Lukaszewska 1997). In this experiment, the regeneration potential of many explant types was examined,

such as shoot bases hidden in the old bulb, lower leafless shoot parts, receptacles and leaves above the inflorescence. All types of explants were subcultured in the same nutrition medium under light or dark conditions. The most efficient and rapid bulblet formation was achieved on explants taken from leafy shoot segments under both light and dark conditions (84 and 95%, respectively). Lukaszewska *et al*. (1998) showed a very high percentage of regeneration from leafy shoot parts in the same medium as described above. These authors also analysed the correlation between the abscisic acid content in the "mother" tissue and the subsequent regeneration ability of *F. imperialis*. Regeneration was more efficient from stem explants (84%) containing the lowest amount of free abscisic acid and higher in leafy stem parts (64%) compared to leafless stems.

Petals have been shown to be a reliable explant type for direct bulblet regeneration of *F. imperialis* (Mohammadi-Dehcheshmeh *et al*. 2008). In this work, petal explants of different stages before (green closed flowers) and after nectar secretion (red closed flowers) were used and cultured on B5 basal medium supplemented with two auxins, IAA and NAA (0.6 and 0.4 mg/l, respectively) and two concentrations of BA (0.1 or 1.0 mg/l). Direct regeneration was higher on medium with a higher BA concentration in all explant types, while bulblet frequency was higher with a lower concentration of BA. Direct bulblet regeneration was activated by removing plant growth regulators from the culture medium. In previous work, indirect bulblet formation was reported when petals were constantly subcultured on medium with plant growth regulators. The authors suggest that the constant presence of growth regulators increased the internal hormonal levels and blocked the direct regeneration pathway. Activated callus production and an indirect regeneration pathway were observed (Mohammadi-Dehcheshmeh *et al*. 2007). Petals taken from red closed flower buds, after nectar secretion, presented a higher morphogenetic response than green closed petals. It was also noted that the regeneration of bulblets started at different parts of the petals, depending on petal stage. From red closed flowers, bulblet formation occurred from the place of nectar secretion, while the edges of petals produced bulblets on explants of green closed flowers which were without nectar secretion. These results might indicate that the sugar in the nectar could positively affect bulblet regeneration in *F. imperialis*.

3. Zygotic embryo culture

There are some reports that large numbers of bulblets of several fritillaries can be produced using mature (Subotić *et al.* 2004) or immature embryo culture (Özcan *et al*. 2007). This type of culture can be very useful for bulb production of geophytes since bacterial and fungal contamination is very common in the culture of bulb scales and other types of explants (Ziv and Lilien-Kipins 2000). Also, zygotic embryo culture is very useful for propagation of endemic and endangered species whenever we need to preserve plant biodiversity. Zygotic embryos of endemic *F. meleagris* were cultured on MS medium supplemented with thidiazuron (TDZ, 0.1-1.0 mg/l) and numerous bulblets formed (**Fig. 1A**). These bulbs could be multiplied for many years in medium of the same composition, but also used as explants in bulb segment culture. *In vitro* formed bulbs of *F. meleagris* cut in half have great morphogenetic potential and numerous bulbs can be formed in MS medium supplemented with TDZ (**Fig. 1B**).

In zygotic embryo culture of *F. alburgyana* and *F. whittalii*, the largest number of bulbs was obtained on MS media supplemented with NAA and BA (0.25 and 4.0 mg/l, respectively) and medium supplemented with the N6 mineral formulation (Chu *et al*. 1975) containing 2 mg/l 2,4-D. Bulblets formed on calli, and over 100 bulblets/explant could be produced following the described protocol (Özcan *et al*. 2007).

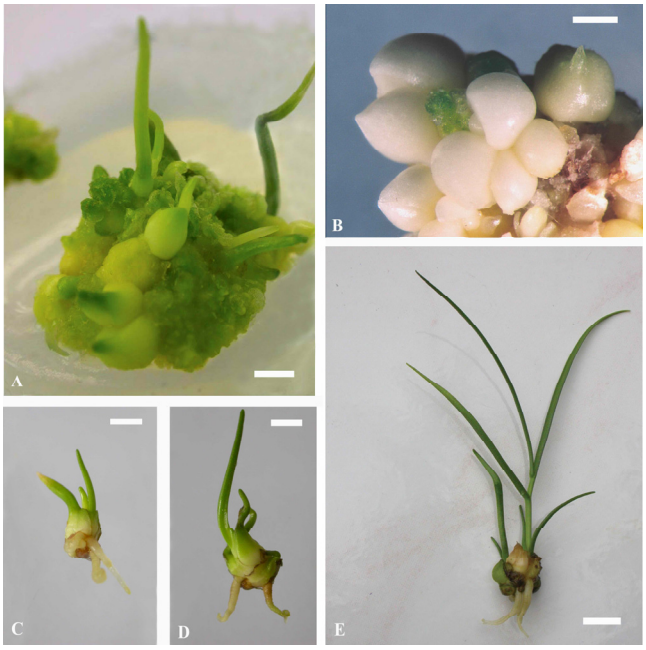

Fig. 1 Plant regeneration of *F. meleagris* by bulb production. (**A**) Bulb formation on MS medium with 0.1 mg/l TDZ in zygotic embryo culture, bar 10 mm (**B**) Bulbs formed on MS medium with 0.5 mg/l TDZ in bulb scale culture, bar 10 mm (**C**) Bulb after cold treatment for 6 weeks at 4°C, bar 10 mm. (**D**) Rooted and sprouted plantlet after 6 weeks of cold treatment and 7 days at room temperature, bar 10 mm (**E**) Fully developed plantlet before planting in greenhouse conditions, bar 10 mm.

Somatic embryogenesis

Plant regeneration of fritillaries via somatic embryogenesis has many advantages. Established protocols allow fast and effective ways for the production of large numbers of somatic embryos which could be separated from explants very easily and regenerated to whole plantlets (**Table 2**). Somatic embryos can be obtained directly on explants or indirectly with a callus interphase. Every plantlet that originated from the somatic embryos of the same explant had the same characteristics as the mother plant. Up to now plant regeneration by somatic embryogenesis has been reported for eight *Fritillaria* species. The main explant types for the induction of calli and somatic embryos are bulbs, bulb scales, stems, petals, roots, zygotic embryos or *in vitro* formed bulbs. Very often, somatic embryogenesis is simultaneously induced with organogenesis (bulb formation) with the same experimental conditions and explants (Hao *et al*. 1995; Özcan *et al*. 2007). This phenomenon is very common in other monocotyledonous plant species (Jevremović *et al*. 2006). There were no information on true-to-typeness of regenerated fritillary plantlets by somatic embryogenesis and organogenesis. Genetic stability and uniformity assessment of irises regenerated by both processes showed that plantlets have a diploid chromosome number and high fidelity screened by RAPD analysis (Jevremović *et al*. 2009, 2010b).

1. Bulb culture

The first results on the induction of embryogenic calli of fritillaries were reported by Hao *et al.* 1982). Callus was obtained from scale fragments of *F. pallidiflora* cultured on MS medium supplemented with 2,4-D and KIN (1.0 and 0.1 mg/l, respectively). Somatic embryo and bulb production from the same calli were noted for the first time in a study by Hao *et al*. (1989). Cytomorphological observations revealed that bulblets were formed from *F. pallidiflora* calli in two ways: 1) specialised cells in the calli divide and develop into buds and bulblets and 2) specialised embryogenic cells from the epidermis of the calli can divide and

develop into embryoids which grow further into bulblets. Morphological observations have also revealed differences between embryos and adventitious buds (Hao *et al*. 1989). Wang *et al*. (1989) reported that embryogenic calli of *F. pallidiflora* can also be induced on MS media supplemented with NAA (1.0 mg/l) and KIN (0.5 mg/l) or BA (2.0 mg/l). Following treatment with low temperature (0-4°C) for 4 weeks, and subsequent subculturing on MS medium supplemented with IAA and KIN (0.1 and 0.5 mg/l, respectively), calli gave rise to plantlets by somatic embryogenesis after 30 days. Cytohistological observations in the same study showed that somatic embryos of *F. pallidiflora* have a single cell origin. The chromosome number of regenerated plantlets was diploid. This is important since previous results showed that 2,4-D in callus culture of *F. pallidiflora* can induce changes in chromosome number. A higher frequency of polyploid cells has been recorded on medium with 2,4-D and KIN than with IAA and NAA (Wang *et al*. 1987). In addition, the frequency of diploid cells in a callus decreases with the age of culture and the callus becomes mixoploid. As a final result, differentiation of calli declines with the accumulation of chromosomal abnormalities (Wang *et al*. 1990).

Embryogenic calli of *F. ussuriensis* could be induced from bulb segments on MN medium supplemented with 2,4-D and KIN (2.5 and 1.0 mg/l, respectively). After subculturing an embryogenic callus on N6 medium with KIN (1.0 mg/l), somatic embryos were formed. They originated from both the surface and the inside of the embryogenic callus and directly on explants (Wu and Tang 1992b).

Somatic embryogenesis of *F. meleagris* has achieved in culture of bulblet scale sections of *in vitro* formed bulbs (Petrić *et al*. 2011). Using *in vitro* formed bulbs as the starting explants for induction of somatic embryogenesis has many advantages, such as avoidance of contaminations when using bulbs from nature and also, reduction of natural population destruction of *Fritillaria* sp. (Witomska and Lukazewska 1997). Somatic embryos of *F. meleagris* are formed on MS media supplemented with sole 2,4-D (0-10.0 mg/l) or TDZ (0-2.0 mg/l). The induction of somatic embryogenesis using 2,4-D as a sole growth regulator is often for monocotyledons species but usage of TDZ is rare and new for somatic embryogenesis induction of *Fritillaria* sp. (Petrić *et al*. 2011).

2. Leaf base culture

Direct somatic embryogenesis, without callus formation, was induced in a culture of leaf base explants excised from *in vitro* grown shoots of *F. meleagris* (Subotić *et al.* 2010). Leaf explants were cultured on MS medium supplemented with various concentration of 2,4-D or KIN (0-10.0 mg/l). The best results were obtained with 2,4-D (0.1 mg/l) with 93% of explants responding and the highest average number of somatic embryos (ca.10 somatic embryos/explant) (**Fig. 2C**). Somatic embryogenesis can also be induced on medium supplemented only with KIN (**Fig. 2D**). The best results were on media supplemented with KIN (0.1 or 0.5 mg/l) resulting in 5.0 somatic embryos/explant. Somatic embryogenesis was also observed on medium without plant growth regulators which can be a consequence of the carry-over of plant regulators (mainly TDZ) from previous subcultures. This plant regulator was shown as a potent inducer of morphogenesis for *F. meleagris* (Subotić *et al.* 2004; Nikolić *et al*. 2006; Petrić *et al*. 2011). Somatic embryogenesis induction in this species was evaluated by observing leaf explants under a scanning microscope after different times after culture initiation. The first morphological changes were observed on leaf explants after 7 days (early globular stadium of somatic embryogenesis) and somatic embryo maturation was evident after 28 days. This observation revealed that somatic embryos originated directly from the epidermal and sub-epidermal layers of leaf explants (Subotić *et al.* 2010). This method for the induction of somatic embryogenesis can be useful for other fritillaries since the loss of morphogenetic potential during prolonged culture has been reported for many species.

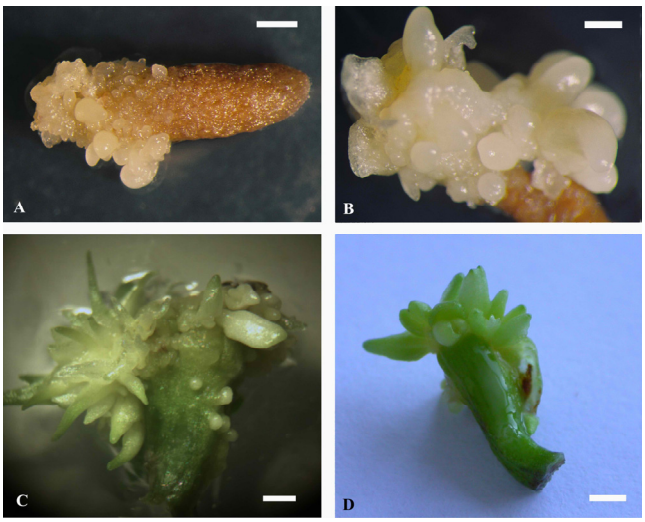

Fig. 2 Somatic embryogenesis induction in tissue culture of *F. meleagris*. (**A**) Induction of somatic embryogenesis on MS medium with 2,4-D and KIN (1.0 mg/l, each), after 3 weeks of zygotic embryo culture, bar 1 mm (**B**) Somatic embryos formed on MS medium with 2,4-D and KIN (1.0 mg/l, each) after 8 weeks of zygotic embryo culture, bar 1 mm. (**C**) Somatic embryos induced on MS medium with 0.1 mg/l 2,4-D, in leaf base culture, bar 1 mm (**D**) Induction of somatic embryogenesis on MS medium with 0.1 mg/l KIN, in leaf base culture, bar 1 mm.

3. Stem and inflorescence parts culture

Bulb regeneration and somatic embryogenesis were achieved simultaneously in a young stem culture of *F. sinica*. Explants were cultured on MS medium supplemented with two auxins (2,4-D, NAA) and two cytokinins (KIN and BA) and calli were formed. Cultures were transferred to MS medium with NAA and BA (1.0 and 0.5 mg/l, respectively) and subcultured every 30-40 days; the regeneration of plantlets by somatic embryogenesis and organogenesis was obtained (Hao *et al.* 1995).

Stems as starting explants were used for the induction of somatic embryogenesis in *F. ussuriensis* (Tang *et al.* 1996). These results suggest that the mineral composition is very important for the morphogenetic response and the way of propagation *in vitro*. MS medium promoted the formation of adventitious buds while N6 medium promoted the induction of somatic embryos. The ability for bud formation decreased with an increase of sucrose in the medium, but somatic embryogenesis increased under these conditions. Also, as mentioned previously, the rates of shoot and somatic embryo formation decreased with protracted time in tissue culture (Tang *et al.* 1996).

Mohammadi-Dehcheshmeh *et al.* (2007) used petal explants of wild *F. imperialis* for the induction of *in vitro* somatic embryogenesis. This explant could be a very reliable plant material for the micropropagation of fritillaries because of the low percentage of infection compared to other explant types. These protocols can be applied to other endangered or endemic fritillaries without destroying them in their natural habitat. Besides the influence of different plant growth regulators, the authors examined effects of cold pre-treatment of green flower buds (4 weeks at 4°C) and light conditions on the induction of embryogenic calli. It was demonstrated that cold pre-treatment had an inhibitory effect on callus formation as well as somatic embryo production. Petals cultured on B5 medium supplemented with IAA, NAA and BA (0.4, 0.6 and 0.1 mg/l, respectively) gave the best morphogenetic response with 56% of explants forming calli. Calli formed on this medium composition were embryogenic as observed 120 days after the initiation of culture. These results suggest that a low con-

centration of BA in combination with these two auxins is crucial for the induction and regeneration of indirect somatic embryogenesis of *F. imperialis*. Somatic embryogenesis of this species is not affected by light conditions (Mohammadi-Dehcheshmeh *et al.* 2007).

4. Root culture

A high frequency (95%) of embryogenic callus induction of *F. ussuriensis* was observed in root tip culture (Feng *et al*. 2009). Medium supplemented with the ER (Eriksson 1965) mineral formulation and 2,4-D, IAA and BA (0.1, 2.0 and 1.0 mg/l, respectively) was shown to be the most suitable for the induction of embryogenic calli and embryogenic cell complexes. The best medium for somatic embryo formation and further plantlet development was ER with IAA and BA (0.1 and 1.0 mg/l, respectively) with 100% of embryos converting into plantlets after 40 days of culture (Feng *et al*. 2009).

5. Zygotic embryo culture

Zygotic embryo culture is not very often used for rapid propagation of fritilaries since with this type of culture you are working with different genotypes at same time, but can be very useful method for biodiversity preservation. Somatic embryogenesis was reported for the first time by Subotić *et al.* (2004) in zygotic embryo culture of *F. meleagris*, after 6 weeks of culture on MS medium supplemented with 2,4-D or TDZ (1.0 mg/l, each). Induction of somatic embryogenesis was observed in both cases, but on zygotic embryos cultured on MS medium containing TDZ, direct regeneration was observed, while 2,4-D induced the formation of embryogenic calli first (**Fig. 2A, 2B**), followed by somatic embryo formation (Subotić *et al*. 2006). The induction of bulblet and somatic embryogenesis on the same explant at the same time has also been reported for other *Fritillaria* species (Özcan *et al*. 2007).

Androgenesis

To date, there is only one report on androgenesis in fritillaries. Plant regeneration of haploids was achieved in anther culture of *F. ussuriensis* (Du *et al*. 1986). Several mineral formulations and hormone combinations were tested. The best frequency of callus induction was 0.2% on MS medium. The callus induction frequency was nine-fold higher with the addition of 2,4-D, BA (0.5 mg/l, each), lactoalbumin (1000 mg/l) and sucrose (6%). The obtained callus was further cultured on several media for the regeneration of bulbs with different concentrations of auxin and cytokinin as well as variable macroelement levels. After 10 days, androgenic calli started to proliferate and bulblets regenerated on the surface of the calli. The best results in terms of sprouting and rooting of pollen-derived bulbs were achieved on half-strength MS medium without growth regulators. One-quarter of the root tip cells had 12 chromosomes, which means that they were haploids. In addition to haploid cells, diploid (31%), tetraploid (11%) and aneuploid (35%) cells were observed. These results indicate that during tissue culture of *F. ussuriensis*, spontaneous doubling of chromosomes occurs (Du *et al*. 1986; Sun and Wang 1991).

Breaking dormancy and acclimatisation of plants

The normal development periodicity of most fritillaries may be modified by the onset of dormancy at low temperature (Zhu *et al*. 1980). Many studies show that most *Fritillaria* bulbs and seeds must be subjected to a low-temperature treatment to break dormancy, and to achieve sprouting and germination (Chen *et al*. 1993; Tang *et al*. 2006; Yu *et al*. 2009). Chen *et al*. (1993) showed that physiological dormancy of seeds was released after chilling for 73-91 days at 5°C. After stratification, the zygotic embryo is activated with optimum germination at 25°C (Yu *et al*. 2008). Seeds of *F. meleagris* need low temperatures (10°C) for at least 10 weeks; subsequently, germination starts and can be continued at 25°C. Seeds of *F. thunbergii* require low temperatures (50 days at 8-10°C and 80 days at 3-5°C) for breaking dormancy (Gao *et al*. 1997a, 1997b, 1997c). Under greenhouse conditions, the highest flowering percentage of *F. meleagris* was obtained after cooling at 5°C for 13-17 weeks (Van Leeuwen and Dop 1990). The effect of cooling temperature and duration on flowering and stem length was studied in *F. imperialis*, which was forced as a cut flower or as a potted plant (Van Leeuwen *et al*. 2002).

Some fritillaries do not need chilling for morphogenesis induction, breaking dormancy and plantlet development, e.g., *F. camtschatcensis* (Otani and Shimada 1997). Okawa and Katijima (1998) showed that the optimum conditions for increasing bulb weight, leaf emergence and rooting of *F. camtschatcensis* bulblets were 8 weeks of storage at 20°C in the dark. NAA, KIN and EB were tested for their effects on leaf emergence and rooting in *F. camtschatcensis* bulblets. The combination of NAA and KIN (1.0 and 0.1 mg/l, respectively) was optimal for bulblet growth, while the same MS medium supplemented with EB (0.01 mg/l) promoted the rooting of bulblets (Ohkawa and Nishino 1999). The sucrose concentration in the culture medium has been found to be very important for leaf emergence and rooting of bulbs of *F. camtschatcensis*. Sucrose (30-60 g/l) was effective in increasing bulblet growth; 60 g/l was suitable for leaf emergence while 60 and 120 g/l were suitable for bulblet rooting. Polyethylene glycol was very effective for bulblet rooting, but not for leaf emergence (Okawa and Nishino 2000). Also, during morphogenesis induction in tissue culture of some fritillaries, a low temperature pre-treatment is not necessary or may have an inhibitory effect. In tissue culture of *F. imperialis*, the highest regeneration ability in both investigated years was observed after bulb storage at 30°C in comparison to 22°C and 4°C. In this case, low temperature during bulb storage decreased the efficiency of micropropagation (Lukaszewska *et al*. 1998). Cold pretreatment of petals from green flower buds has an inhibitory effect on callus formation and somatic embryogenesis in *F. imperialis* (Mohammadi-Dehcheshmeh *et al*. 2007).

The effect of low temperature on dormancy breaking of *in vitro* regenerated bulbs has been reported for many bulbous plants such as lily (Shin *et al*. 2002; Langens-Gerrits *et al*. 2003), *Lachenalia* (Slabbert and Niederwieser 1999), tulip (Lambrechts *et al.* 1994) and *Allium* (Specht and Keller 1997; Yamazaki *et al*. 2002). In many cases, if the low temperature treatment is extended (duration depends on species), sprouting, growth and formation of leaves are faster and better. Further prolongation of low-temperature treatment has no effect on breaking dormancy.

The bulbs of many fritillaries formed in culture *in vitro* are also dormant, and they require chilling to break dormancy as well (Li and Qin 1987; Yu *et al*. 1994; Gao *et al*. 1997a; Paek and Murthy 2002; Nikolić *et al*. 2008).

Callus or bulb-derived bulblets of *F. thunbergii* can normally be induced at 18-23°C. Plant regeneration from these bulblets is difficult unless they are treated with low temperature (2-15°C) followed by culture in the light at room temperature (Sun and Wang 1991). Bulblet formation from cultured scale segments of *F. thunbergii* was promoted by dry (2-4 weeks) or moist storage (4-6 weeks) of mother bulbs at 10°C before the excision of explants. Bulb scales taken after 6 weeks of cold, moist storage showed better morphogenetic response than scales taken from dry storage bulbs (Yu *et al*. 1994). To overcome dormancy, the bulblets of *F. thunbergii* were harvested at the end of the culture period and kept at 5°C for 5 weeks. Leaf emergence from chilled bulblets after transplantation in soil depended on the chilling period, the concentration of sucrose in the medium, but also on bulblet size (Paek *et al*. 1996). Leaf emergence was better after planting bigger-sized bulblets (10 mm) which were produced in 3% sucrose medium compared to small bulblets obtained on medium supplemented with higher concentrations of sucrose (≥5%). After cold treat-

ment, these bulblets were potted and 100% of them sprouted within 5 weeks of transplantation (Paek *et al*. 1996; Paek and Murthy 2002).

Successful plant regeneration from callus-derived bulblets of *F. pallidiflora* was performed after bulb storage at 4-10°C for 40 days. After this cold treatment, bulblets were transferred to MS media with the addition of IAA and KIN (0.1 and 0.5 mg/l, respectively) where seedlings, roots and complete plants regenerated (Sun and Wang 1991). Li *et al*. (2003) concluded that embryoids of *F. cirrhosa* treated at 0-5°C for 40 days showed a better germination rate when cultured on MS medium without hormones compared to standard conditions. Pre-incubation of *F. anhuinensis* callus cultures at 5°C for 30-40 days was suitable for successful bulblet formation on MS medium supplemented with NAA and KIN (2.0 mg/l each), 5% sucrose and 5 g/l activated charcoal (Xue *et al.* 2008).

The rooting rate after six weeks of chilling of *F. meleagris* bulbs was two-fold higher when compared to standard conditions (Nikolić *et al*. 2008; **Fig. 1C, 1D**). Shoot and root length of chilled bulblets was greater and after transfer to the greenhouse, and chilled plantlets showed a greater survival rate compared to control plants (**Fig. 2E**). Metabolism of sugars and starch is closely linked to different stages of tissue culture, dormancy and sprouting of bulbous plants. Total solubile sugars and starch content of *F. ussuriensis* are minimum at callus stage and starch content reached maximum during bulblet expanding stage (Sun 2010). When bulblets are stored at low temperature, there is a breakdown of starch and an accumulation of sucrose from starch hydrolysis (Shin *et al*. 2002). Accumulated sucrose can be utilised by bulbs for sprouting initiation after breaking dormancy, as well as for leaf growth and development of the photosynthetic apparatus. Witomska (2000) showed that bulbs of *F. imperialis* subjected to treatment at 4°C had a greater soluble sugar content compared to those cultivated at 22°C and 30°C. In chilled bulbs of *F. meleagris*, accumulation of glucose and fructose significantly increased after cold treatment (4°C for 6 weeks) but there was no great increase in sucrose content (Nikolić *et al*. 2008). Changes in superoxide dismutase (SOD) activity and isoenzyme profiles of *in vitro*-derived *F. meleagris* bulbs in response to cold treatment (4°C) before and after 6 weeks of cold treatment were reported in Jevremović *et al*. (2010b). SOD activity in bulbs initially decreased, but then rapidly increased 7 days after cold treatment. Four isoforms of SOD are active in bulbs of *F. meleagris* under standard and chilled conditions. Two isoforms are MnSOD while the two low-weight forms are FeSODs. Seven days after cold treatment, two FeSODs were still active, but only one mitochondrial MnSOD showed sustained activity. The presence and activity of SOD enzymes are indicators of oxidative stress induced by cold treatment, but are also indicators of developmental changes which occur during the process of dormancy breaking in bulbs (Jevremović *et al*. 2010b).

METABOLITE PRODUCTION

Many higher plants are major sources of secondary metabolites for the pharmaceutical, agrochemical, flavour and aroma industries (Karuppusamy 2009). *Fritillaria* is, besides *Veratrum*, the most important genus of the *Liliaceae* family which contains biologically active steroid alkaloids (Li *et al*. 2006).

Many fritillaries are traditionally used as herbal remedies in Japanese (Sho *et al*. 1963; Kaneko *et al*. 1981), Chinese (Li *et al*. 1993), Turkish (Rahman *et al*. 2002), Pakistani and south-east Asian folk medicine. The 2005 edition of the Chinese Pharmacopoeia includes nine fritillaries (*F. thunbergii*, *F. chirrhosa*, *F. unibracteata*, *F. przewalski*, *F. delavayi*, *F. ussuriensis*, *F. walujewavii*, *F. pallidiflora* and *F. hupehensis*) as the plant sources for herbal Beimu which is used for its anti-tussive, anti-asthmatic and expectorant properties. From a pharmacophylogenetic point of view, Beimu as a plant source is divided in six groups depending on plant species as well as the geographical origin of plants (Xiao *et al*. 2007). *F. cirrhosa* Beimu, also known in China as Chuanbeimu, was accepted by the Chinese Pharmacopoeia (2005) as a traditional medicine for relieving coughs and eliminating phlegm. The annual output of remedies from this plant covers only some 5% of the market demand (Li *et al*. 2009). The main active constituents in medicinally valuable fritillaries are isosteroidal alkaloids (Orhan *et al*. 2006). The alkaloid content is relatively low, estimated at 0.1-0.4% of the dry weight (Dharmananda 2010). Amongst all compounds found, the majority (72.7%) belong to the class of isosteroidal alkaloids, while the rest are steroidal alkaloids (11.5%) and non-alkaloids (15.8%). Isosteroidal alkaloids isolated from fritillaries are classified into five types according to their structural skeleton: the cevanine, jervinine, veratramine, solanidine and secosolanidine types (Jiang *et al*. 2006). All isosteroidal alkaloids isolated from fritillaries are predominantly of the cevanine type (Rahman and Choudhary 1993, 1997). The cevanine type of alkaloid could be of the D/E *trans* or D/E *cis* cevanine group which can be used in chemotaxonomy research (Xiao *et al*. 2007). Some studies have shown that chemical variation in cevanine alkaloids from fritillaries is geographically dependent (Yu and Xiao 1992; Li *et al*. 1999b). Eight cevanine isosteroidal alkaloids (imperialine, verticine, verticinone, isoverticine, ebeiedine, ebeiednone, ebeienine and hupehenene) and one jervinine type (peimissine) are the major bioactive constituents of beimu (Lin *et al*. 2001).

Recently, *Fritillaria* bulbs also became important because they are very rich in starches which are the main component of the bulbs, comprising approximately 80% of the total biomass (Gao *et al*. 1999). Starches as the most important polysaccharide reserve in higher plants and have been used for centuries and studied from different plant sources like potato, rice, corn and wheat. Wang *et al*. (2005a, 2005b, 2006) studied three *Fritillaria* starches for their use in food and medicine. They showed that *F. thunbergii* and *F. ussuriensis* starch granules ranged in size from 5 to 30 μm, while *F. pallidiflora* starch granules ranged from 5 to 40 μm. It is also revealed that the crystal type of the three *Fritillaria* starches was the characteristic B-type, as seen in *F. hupehensis*, which was in agreement with the crystal type of potato starch; while *F. cirrhosa* starches showed the C_B-type pattern. Based on the shape, size, hilum and striation on the starch grain, some fritillaries can be distinguished (Liu *et al*. 1997).

Alkaloid production of fritillaries *in vitro*

Plant tissue culture plays a vital role in the search for alternatives to the production of desirable medicinal compounds from plants (Karuppusamy 2009). The capacity of several fritillaries to accumulate alkaloids in tissue culture has been reported in many papers (**Table 3**).

The first results date from 1992, when several research groups reported qualitative and quantitative analysis of alkaloids in cultured *F. unibracteata* (Cai *et al*. 1992; Gao *et al*. 1992; Xu *et al*. 1992). In work reported by Zhu e*t al*. (1992), the types of accumulated alkaloids in cultured bulbs were similar to those found in bulbs collected in the wild, but the contents of alkaloids were higher. Both types of bulbs had positive effects on relieving cough and removing phlegm in mice (Zhu *et al*. 1992). MS medium resulted in the highest yield of alkaloids from *F. unibracteata* (Xu *et al*. 1992). In tissue culture of *F. unibracteata*, sugar can substitute sucrose and reduce production costs but also increase the yield of alkaloids by about 22% (Xu *et al*. 1992). Gao *et al*. (1992) reported that the highest growth rate and yield of alkaloids in bulbs of *F. unibracteata* occurred after 50 days of culture. The content of alkaloids was at a high level throughout the culture period and was 1.2 to 1.5-fold higher than in wild bulbs. Cao *et al*. (1992) showed that the highest growth rate and alkaloid content in tissue culture was observed in a shaken culture at 20°C; there was no significant difference between culture in the light and in the dark. The

Table 3 List of *Fritillaria* sp. investigated for alkaloid production by tissue culture.

Species	Culture conditions	Plant tissue	Reference
F. cirrhosa	low temperatures (15°C)	bulb	Li *et al.* 2008
	MS + NAA 0.5 + BA 2.0	callus	Wang *et al.* 2010b
	MS + NAA 0.2 + BA 2.0	cell suspension	Wang *et al.* 2011
F. mellea	MS + NAA 1.0 + BA 3.0	callus	Li *et al.* 2002
F. monatha	MS	bulb	Zhang *et al.* 2007
F. unibracteata	MS	bulb	Xu *et al.* 1992
	MS	bulb	Gao *et al.* 1999
F. ussuriensis	MS + 2,4-D 0.5 + BA 0.2	callus	Zhang *et al.* 2003
	LS + NAA 5.0 + BA 0.5	bulb	Liang *et al.* 2005

BA, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **LS**, medium according to Linsmaier and Skoog, (1965); **MS**, medium according to Murashige and Skoog (1962); **NAA**, α-naphthaleneacetic acid

highest alkaloid yield was achieved at 5% sucrose, while the addition of amino acids was favourable for bulb growth, but not for accumulation of alkaloids (Cai *et al*. 1996). Gao *et al*. (1999) indicated that the content of alkaloids in cultured bulbs of *F. unibracteata* was higher (0.045-0.067%) than in the wild bulb (0.040%). These results indicate that organ culture of fritillaries maintains the compositional stability in tissue and can be used for large-scale production of secondary metabolites. The authors reported that bulbs can be subcultured for six years with a 30 to 50-fold increase in the growth rate when compared to the natural situation (Gao *et al*. 1999).

In bulbs regenerated by tissue culture of *F. pallidiflora*, accumulation of imperialine was recorded. The composition in the roots was more complex than in the other tissues. The content of alkaloids in adventitious roots and bulbs subcultured for eight generations was higher than in those cultivated for 5 years (Tingyao *et al*. 1999).

Alkaloids also accumulate in callus cultures of fritillaries. In many reports, it has been shown that the content of alkaloids in calli was higher than in fresh bulbs. The alkaloid content in fresh bulb culture and callus culture of *F. mellea* were compared by Li *et al*. (2002). The best conditions for callus induction was medium supplemented with NAA and BA (1.0 and 3.0 mg/l, respectively) at 22°C. The content of alkaloids in callus cultures was higher than in bulbs and was highest at about the 20th day after callus induction (Li *et al*. 2002). Callus growth and the alkaloid content of *F. ussuriensis* were compared in different nutritional media, and the results demonstrated that the content of alkaloids was greatest when cultures were grown on MS medium with the addition of 2,4-D and BA (0.5 and 0.2 mg/l, respectively). When calli were grown on MS medium supplemented with NAA and BA (3.0 and 0.5 mg/l, respectively), the content of alkaloids in the calli was higher than in dried herbs of *F. ussurensis* and *F. thunbergii* (Zhang *et al.* 2003). On the other hand, Liang *et al*. (2005) showed that solid LS medium was better than others for improving the yield of alkaloids from *F. ussuriensis*. The highest yield (1.62 mg/l) was achieved on solid LS medium supplemented with NAA, BA (0.5 and 1.0 mg/l, respectively), ribavirin and sucrose (5.0 mg/l each) after 60 days of culture. The yield and the content of alkaloids showed no significant increase in liquid LS medium under the same conditions, but the liquid cultures stimulated bulb production (Liang *et al*. 2005). MS medium was favourable for alkaloid accumulation from bulbs of *F. monatha*, and the alkaloid content peaked after 60 days (Zhang *et al*. 2007). Alkaloid production in callus culture with the selection of high-yield cell lines of *F. monatha* was reported by Zhang *et al*. (2007). These results suggest that the culture proliferation rate reached a maximum on the 50th day of culture, while alkaloid production peaked 10-20 days after the start of culture. High alkaloid content cell lines can be visually selected based on colour (Zhang *et al*. 2007).

Studies on the histochemical localisation of alkaloids in wild and tissue-cultured *F. cirrhosa* have shown that alkaloids in bulbs mainly accumulate in parenchyma cells, while alkaloids in calli mainly accumulate around the cell wall (Yang *et al*. 2008).

Recently, Wang *et al*. (2011) reported alkaloid production by mass suspension culture of *F. cirrhosa*. Alkaloid accumulation in cell mass was higher than in commercial and wild bulbs. The optimal culture medium was MS with addition of NAA and BA (0.2, 2.0 mg/l, respectively).

CONCLUDING REMARKS

The genus *Fritillaria* from the *Liliaceae* family includes bulbous species that are cultivated as ornamental cut or garden plants. They are also important medicinal plants and are a source of valuable alkaloids. Classical propagation is limited since production of vegetative bulbs is very poor and seeds have a low germination rate. Research on tissue culture of fritillaries was established more than 30 years ago and was concerned with medical species cultivated in China and ornamental species from Europe. The vegetative generation of new plants can be achieved by bulb formation and/or somatic embryogenesis and androgenesis. Most studies investigated micropropagation by bulb formation. Different initial explants i.e. bulb scales, bulb segments (transverse or vertical cuts) or whole bulbs were used as explants for *Fritillaria* micropropagation. To avoid problems with infection, other explant types have recently been used, such as leaf, stem, petals or *in vitro* formed bulbs. For inorganic nutrition, MS usually gives the best results and is used most often. Calli, somatic embryos and bulblets could be induced at different concentrations of auxins and cytokinins. The range of different plant regulators which could induce a morphogenetic response varied for different regulators as well as plant species. Optimal concentrations for most growth regulators were between 0.1 and 1 mg/l. The exposure to low temperatures is very important for many fritillaries in different phases of tissue culture. For some species, the multiplication rate can be increased when initial explants are stored under low temperature conditions for several weeks before morphogenesis induction *in vitro*. Also, low temperature treatment is essential for dormancy breaking of *in vitro* formed bulbs in almost all fritillaries The duration and range of chilling temperatures are species-dependent, but temperatures from 2-15°C for 4 to 12 weeks are very effective for most fritillaries Protocols for the propagation of many fritillaries by somatic embryogenesis using several initial explants have been established. This type of regeneration is very effective and successful for some species. Alkaloid accumulation during tissue culture of medicinally valuable fritillaries was confirmed in organ, callus culture and cell suspensions. The yield of alkaloids during *in vitro* cultivation could be higher than in plants cultivated or collected from nature.

In this review, these summarised results report on the basic conditions for the *in vitro* induction of morphogenesis of fritillaries, which are valuable ornamental and/or medicinal plants. Many of the successful protocols reported here may be useful for the mass production of endangered plants without destroying them in their natural habitat.

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REFERENCES

* in Chinese with abstract in English

Alp Ş, Arslan N, Koyuncu M (2009) Established forms of *Fritillaria imperialis* L. – A naturally growing species in Turkey. *Pakistan Journal of Botany* **41 (4)**, 1573-1576

Badfar-Chaleshtori S, Shiran B, Kohgard M, Mommeni H, Hafizi A, Khodambashi M, Mirakhorli N, Sorkheh K (2012) Assessment of genetic diversity and structure of Imperial crown (*Fritillaria imperialis* L.) populations in the Zagos region of Iran using AFLP, ISSR and RAPD markers and implications for its conservation. *Biochemical Systematics and Ecology* **42**, 35-48

Cai Z, Gao S, Xu D, Zhu D (1992) Effect of different cultural conditions and methods on the growth of cultured bulb of *Fritillaria unibracteata* Hsiao et.K.C.Hsia. *Journal of China Pharmaceutical University* **6**, 367-369*

Cai Z, Zhu D, Tao J, Gao S, Xu D (1996) The effects of concentration of sucrose and addition of aminos on the growth of cultured bulbs of *Fritillaria unibracteata*. *Journal of China Pharmaceutical University* **27 (1)**, 1-3*

Carasso V, Hay FR, Probert RJ, Mucciarelli M (2011) Temperature control of seed germination in *Fritillaria tubiformis* subsp. *moggridgei* (*Liliaceae*) a rare endemic of the South-west Alps. *Seed Science Research* **21 (1)**, 33-38

Chauhan RS, Nautiyal MC, Vashistha RK, Prasad P, Nautiyal AR, Kumar A, Teixeira da Silva JA (2011) Morpho-biochemical variability and selection strategies for the germplasm of *Fritillaria roylei* Hook (*Liliaceae*) - an endangered medicinal herb of western Himalaya, India. *Journal of Plant Breeding and Crop Science* **3 (16)**, 430-434

Chen Y, Zhang J, Li XE (1993) Temperature requirements for afterripening of the embryo of *Fritillaria cirrhosa* Don.f. *China Journal of Chinese Materia Medica* **18 (5)**, 270-272*

Chen UC, Tai CD, Chen CC, Tsay HS (2000) Studies on the tissue culture of *Fritillaria hupehensis* Hsiao et K. C. Hsia III. Influence of medium component and light treatment on *in vitro* rooting and acclimatization of bulblet. *Journal of Agricultural Research of China* **49 (4)**, 39-47*

Chu CC, Wang CC, Sun CS, Chen H, Yin KL, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica* **18**, 659-668

Corneanu GC, Popescu GG (1981) Distributional and anatomical studies on *Fritillaria* (*Liliaceae*) in Romania. *Willdenowia* **11**, 307-315

Dharmananda S (2010) Institute for Traditional Medicine, Portland, Oregon, USA. Available online: http://www.itmonline.org/arts/fritillaria.htm

Du LG, Hou YH (1985) Plant regeneration from immature embryo of a hybrid of species cross of *Fritillaria*. *Chinese Herbs* **2**, 1*

Du LG, Hou YH, Chang WC, Zhao YH, Li AS, Shao QQ, Yang ZT, Chen ZG (1986) Induction of pollen plant and clone establishment of *Fritillaria ussuriensis* Maxim. *Acta Genetica Sinica* **14**, 262-265*

Erickson T (1965) Studies on the growth measurements of cell cultures of *Haplopappus gracilis*. *Physiologia Plantarum* **18**, 976-993

Fast G (1981) *Orchideen Kultur*, Verlag Eugen Ulmer, Stuttgart, Germany, p 222

Feng Y, Gu DZ, Wang YP, Jiang YT (2009) Establishment of *in vitro* somatic embryogenesis and plant regeneration system of wildly *F. ussuriensis* Maxim. *Seed* **1**, 32-36*

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158

Gao S, Zhu D, Cai Z, Xu D (1992) Studies on the characteristics of growth and the content of alkaloids in bulb culture of *Fritillaria unibracteata. Journal of China Pharmaceutical University* **3**, 144-147*

Gao SL, Zhu DN, Cai ZH, Jiang Y, Xu DR (1999) Organ culture of a precious Chinese medical plant – *Fritillaria unibracteata*. *Plant Cell, Tissue and Organ Culture* **59**, 197-201

Gao W, Li Z, Xiao P, Yu S (1997a) Diastase and reducing sugar in *Fritillaria thunbergii* Miq. during the process of dormancy relieving with low temperature. *China Journal of Chinese Materia Medica* **22 (8)**, 467-468*

Gao W, Li Z, Xiao P (1997b) Isoesterase assay over the course of dormancy relieving with low temperature in the seed of *Fritillaria thunbergii* Miq. by electrophoresis. *China Journal of Chinese Materia Medica* **22 (6)**, 13-14*

Gao W, Li Z, Xiao P (1997c) Electrophoresis assay on isoperoxidase in the course of seed dormancy relieving for *Fritillaria thunbergii* Miq. at two different low temperatures. *China Journal of Chinese Materia Medica* **22 (5)**, 20-21*

Hao Y, Li M, Qi L (1989) The production of *Fritillaria pallidiflorae* clone and its adventitious bud and its embryogenesis. *Acta Botanica Boreali-occidentalia Sinica* **4**, 233-239*

Hao YR, Li MS, Wu YW (1982) Callus induction and plant regeneration in tissue culture of *Fritillaria pallidiflora*. *Acta Botanica Boreali-occidentalia Sinica* **2**, 38-43*

Hao Z, Zebin C, Yang L (1995) Organ regeneration and somatic embryogenesis from young stems of *Fritillaria sinica*. *Journal of West China University of Medical Science* **18 (1)**, 33-37*

Jevremović S, Subotić A, Radojević Lj (2006) *In vitro* morphogenesis of dwarf irises. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II) Global Science Books, Isleworth, UK, pp 551-557

Jevremović S, Subotić A, Trifunović M, Nikolić M, Radojević Lj (2008) *In vitro* plant regeneration of *Iris pseudopallida.* In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol V), Global Science Books, Isleworth, UK, pp 250-252

Jevremović S, Stanišić M, Ćalić-Dragosavac D, Raspor M, Ninković S, Subotić A, Trifunović M, Petrić M (2009) Clonal fidelity of *Iris sibirica* plantlets regenerated by culture *in vitro*. In: *IV Congress of Serbian Genetic Society*, 1-5 June 2009, Tara, Serbia. Book of abstracts, p 140

Jevremović S, Petrić M, Živković S, Trifunović M, Subotić A (2010a) Superoxide dismutase activity and isoenzyme profiles in bulbs of snake's head fritillary in response to cold treatment. *Archives of Biological Sciences* **62 (3)**, 553-558

Jevremović S, Ćalić D, Subotić A, Trifunović M, Petrić M, Bohanec B (2010b) Ploidy variation and flowering of dwarf iris derived from tissue culture. *Acta Horticulturae* **855**, 153-158

Jiang Y, Li P, Li HJ, Yu H (2006) New steroidal alkaloids from the bulb of *Fritillaria puqiensis*. *Steroids* **71**, 843-848

Joshi SK, Dhar U, Andola HC (2007) *In vitro* bulblet regeneration and evaluation of *Fritillaria roylei* Hook. - a high value medicinal herb of the Himalaya. *Acta Horticulturae* **756**, 75-83

Kamari G, Phitos D (2006) Karyosystematic study of *Fritillaria messanensis* (*Liliaceae*). *Willdenowia* **36**, 217-233

Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *Journal of Medicinal Plants Research* **3 (13)**, 1222-1239

Kaneko K, Tanaka M, Nakaoka U, Tanaka Y, Yoshida N, Mitsuhashi H (1981) Camtschatcanidine, an alkaloid from *Fritillaria camtschatcensis*. *Phytochemistry* **20 (2)**, 327-329

Kizil S, Arslan N, Ölmez-Bayhan, Khawar KM (2008) Effects of different planting dates on improving yield of *Fritillaria imperialis* L. and *Fritillaria persica* L. bulbs damaged by small narcissus fly (*Eumerus strigatus* Fallen). *African Journal of Biotechnology* **7 (24)**, 4454-4458

Kukulezanka K, Kromor K, Czastka B (1989) Propagation of *Fritillaria meleagris* L. through tissue culture. *Acta Horticulturae* **251**, 147-153

Lambrechts H, Rook F, Kollöffel C (1994) Carbohydrate status of tulip bulbs during cold-induced flower stalk elongation and flowering. *Plant Physiology* **104**, 515-520

Langens-Gerrits MM, Miller WBM, Croes AF, De Klerk GJ (2003) Effect of low temperature on dormancy breaking and growth after planting in lily bulblets regarded *in vitro*. *Plant Growth Regulation* **40**, 267-275

Li CJ, Qin ZD (1987) The relationship between physiological and biochemical changes and releasing dormancy of *Fritillaria pallidiflora* Schrenk. during low temperature treatment. *Acta Botanica Boreali-occidentalia Sinica* **7 (1)**, 23-28*

Li HJ, Jiang Y, Li P (2006) Chemistry, bioactivity and geographical diversity of steroidal alkaloids from the *Liliaceae* family. *Natural Product Reports* **23**, 735-752

Li P, Ji H, Xu GJ (1993) Studies on the antitussive and expectorant effects of Chinese drug Beimu. *Journal of China Pharmaceutical University* **24 (6)**, 360-362*

Li K, Wu W, Zheng Y, Dai Y, Xiang L, Liao K (2009) Genetic diversity of *Fritillaria* from Sichuan province based on ISSR. *China Journal of Chinese Materia Medica* **34 (17)**, 2149-2154*

Li KQ, Wu W, Zheng YL, Dai Y, Mou L, Liao K (2008) Effect of temperature on physiologic indexes, bulb yielding and total alkaloids contents of *Fritillaria cirrhosa*. *China Journal of Chinese Materia Medica* **33 (16)**, 1948-1951*

Li Q, Ling L, Fu HL, Qing RW, Lan LQ (2003) The influence of low temperature in the further germination of embryoids of *Fritillaria cirrhosa* D. Don. *Journal of Sichuan University* **40 (2)**, 367-370*

Li X, Gao W, Jiang Q, Huang L, Liu C (2011) Study on the morphology, crystalline structure, and thermal properties of *Fritillaria ussuriensis* Maxim. starch acetates with different degrees of substitution. *Starch* **63**, 24-31

Li YF, Yan F, Tang L, Xu Y, Chen F (2002) Studies on condition of tissue culture and accumulation of alkaloids at different period in *Fritillaria mellea*. *Chinese Traditional and Herbal Drugs* **33 (5)**, 77-78*

Liang LY, Cao QF, Qian ZY (2005) Effects of culture conditions on the alkaloid yield of *Fritillaria ussuriensis* Maxim from bulb *in vitro*. *Acta Agriculturae Nucleatae Sinica* **19 (3)**, 175-180*

Lin G, Li P, Li SL, Chan SW (2001) Chromatographic analysis of *Fritillaria* isosteroidal alkaloids, the active ingredients of Beimu, the antitussive traditional Chinese medicinal herb. *Journal of Chromatography A* **935**, 321-338

Linsmaier EM, Skoog F (1965) Organic growth factor requirement of tobacco tissue culture. *Physiologia Plantarum* **18**, 100-1270

Liu H, Ding K, Li P, Zhang C, Xu G (1997) Microscopic identification of beimu grown in Yunnan province. *China Journal of Chinese Materia Medica* **22 (12)**, 716-719*

Liu Y, Wang W, Li Z (1996) Experiment on the rapid reproduction of *Fritillaria taipaiensis* P. Y. Li. *China Journal of Chinese Materia Medica* **21 (1)**, 15-7*

Lukaszewska AJ, Witomska M, Bianco J, Barthe P (1998) ABA contents and the regeneration ability of *Fritillaria imperialis* L. cultured *in vitro*. *Acta Physiologiae Plantarum* **20 (3)**, 241-244

Mancuso E, Bedini G, Peruzzi L (2012) Morphology, germination, and storage behaviour in seeds of tuscan populations of *Fritillaria montana* (*Liliaceae*), a rare perennial geophyte in Italy. *Turkish Journal of Botany* **36**, 161-166

Mohammadi-Dehchesmeh M, Khalighi A, Naderi R, Ebrahimie E, Sardari M (2007) Indirect somatic embryogenesis from petal explant of endangered wild population of *Fritillaria imperialis*. *Pakistan Journal of Biological Sciences* **10 (11)**, 1875-1879

Mohammadi-Dehcheshmeh M, Khalighi A, Naderi R, Saradi M, Ebrahimie E (2008) Petal: A reliable explant for direct bulblet regeneration of endangered wild population of *Fritillaria imperialis* L. *Acta Physiologiae Plantarum* **30**, 395-399

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nalawade SM, Sagare AP, Lee CY, Kao CL, Tsay HS (2003) Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Botanical Bulletin of Academia Sinica* **44**, 79-98*

Naohiro N, Kenji W (2006) *Fritillaria tokushimensis* (*Liliaceae*) is thought to be a natural hybrid. *Journal of Phytogeography and Taxonomy* **54**, 35-44 (in Japanese with abstract in English)

Nikolić M, Radojević Lj, Jevremović S, **Trifunović M, Subotić A** (2006) *In vitro* formation of somatic embryos and bulblets of *Fritilllaria meleagris*. In: *4th Balkan Botanical Congress*, *Plant, Fungal and Habitats Diversity Investigation and Conservation*, 20 – 26 June, 2006, Sofia, Bulgaria, p 136 (Abstract)

Nikolić M, Mišić D, Maksimović V, Jevremović S, Trifunović M, Subotić A (2008) Effect of low temperature on rooting rate and carbohydrate content of *Fritillaria meleagris* bulbs formed in culture *in vitro*. *Archive of Biological Sciences* **60 (1)**, 5-6

Okawa M, Kitajima J (1998) Studies on the propagation of *Fritillaria camtschatensis* Ker-Gawl. by *in vitro* culture. I. Characteristics of sprouting, leafiness, flowering and new bulb formation from mother bulb, and bulblet formation from small globule-scale. *Journal of the Japanese Society for Horticultural Science* **67(2)**, 242-248 (in Japanese with abstract in English)

Okawa M, Kitajima J, Nishino K, Okawa N (1999) Studies on the propagation of *Fritillaria camtschatensis* Ker-Gawl. by *in vitro* culture. II. Effect of plant hormones on the growth, leaf emergence, and rooting of bulblets. *Journal of the Japanese Society for Horticultural Science* **68 (1)**, 184-188 (in Japanese with abstract in English)

Okawa M, Nishino K (1999) Effect of temperature and light intensity on growth of the regenerated bulblet of *Fritillaria camtschatensis* Ker-Gawl. *in vitro* culture. *Plant Propagation* **37 (4)**, 243-247

Okawa M, Nishino K (2000) Effects of sucrose and polyethylene glycol on leaf emergence, rooting and bulblet growth of *Fritillaria camtschatcensis* Ker-Gawl. *in vitro* culture. *Environmental Control in Biology* **38 (2)**, 99-103

Orhan I, Sener B, Koyuncu M (2006) Advances in the genus *Fritillaria* L.: Phytochemical and bioactivity approaches. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol IV), Global Science Books, Isleworth, UK, pp 438-448

Otani M, Shimada T (1997) Micropropagation of *Fritillaria camtschatcensis* (L.) Ker-Gawl., "Kuroyuri". *Bulletin of RIAR, Ishikawa Agricultural College* **5**, 39-44

Özcan S, Parmaksiz I, Mirici S, Çöçü S, Uranbey S, Ipek A, Sancak C, Sarihan E, Gürbüz B, Sevimay CS, Arslan N (2007) Efficient *in vitro* bulblet regeneration from immature embryos of endemic and endangered geophyte species in *Sternbergia*, *Muscari* and *Fritillaria* genera. In: Xu Z, Li J, Xue Y, Yang W (Eds) *Biotechnology and Sustainable Agriculture 2006 and Beyond*, Springer, Berlin, Germany, pp 381-383

Paek KY, Yu KJ, Sung NS, Choi IS, Cho JT (1994) Micropropagation through stem, node-bud, shot tip and bulblet scale culture in *Fritillaria thunbergii* Miq. *Korean Journal of Medicinal Crop Science* **2**, 154-161

Paek KY, **Sung NS, Park CH** (1996) Several factors affecting bulblet regeneration from the culture of scale segment and node – bud in *Fritillary* as medicinal bulbous plant. *Acta Horticulturae* **440**, 499-503

Paek KY, Murthy HN (2002) High frequency of bulblet regeneration from bulb scale section of *Fritillaria thunbergii*. *Plant Cell, Tissue and Organ Culture* **68**, 247-252

Petrić M, Subotić A, Jevremović S, Trifunović M (2011) Somatic embryogenesis and bulblet regeneration on shakehead fritillary (*Fritillaria meleagris* L.). *African Journal of Biotechnology* **10 (72)**, 16181-16188

Qiao HL, Ma XF, Li LY (1986) Preliminary report of greenhouse production of bulb of *Fritillaria sichuanica*. *Journal of Traditional Chinese Medicine* **11**, 10-11*

Rahman A, Choudhary MI (1993) Diterpenoid and steroidal alkaloids. *Natural Product Reports* **12**, 361-379

Rahman A, Choundhary MI (1997) Diterpenoid and steroidal alkaloids. *Natural Products Reports* **14**, 191-203

Rahman A, Akhtar MN, Choudhary MI, Tsuda Y, Sener B, Khalid A, Parvez M (2002) New steroidal alkaloids from *Fritillaria imperialis* and their cholinesterase inhibiting activities. *Chemical and Pharmaceutical Bulletin* **50 (8)**, 1013-1017

Seon JH, Paek KY, Gao WY, Park CH, Sung NS (1999) Factors affecting micropropagation of patogen – free stocks in *Fritillaria thunbergii*. *Acta Horticulturae* **502**, 333-337

Shiau YJ, Tai CD, Chen CC, Tsay HS (2000) Studies on the tissue culture of *Fritillaria hupehensis* Hsia et K. C. Hsia I. The induction of bulbscale and embryogenic callus from different source explants. *Journal of Agricultural Research of China* **49**, 29-38*

Shin KS, Chakrabarty D, Paek KY (2002) Sprouting rate, change of carbohydrate contents and related enzymes during cold treatment of lily bulblets regenerated *in vitro*. *Scientia Horticulturae* **96**, 195-204

Sho I, Michiharu, Testuo N (1963) On the alkaloid of *Fritillaria verticillate* WILD. var. Thunbergii BAKER. II. The structure of verticine. *Chemical and Pharmaceutical Bulletin* **11 (10)**, 1337-1340

Slabbert MM, Niederwieser JG (1999*) In vitro* bulblet production of *Lachenalia*. *Plant Cell Reports* **18**, 620-624

Specht CE, Keller ERJ (1997) Temperature requirements for seed germination in species of the genus *Allium* L. *Genetic Resources and Crop Evolution* **44**, 509-517

Subotić A, Jevremović S, Nikolić M, Radojević Lj (2004) Induction of *in vitro* morphogenesis of spp. *Fritillaria*. In: *5th In Vitro Culture and Horticultural Breeding Symposium*, 12-17 September, 2004, Debrecen, Hungary, p 101 (Abstract)

Subotić A, Trifunović M, Jevremović S, Petrić M (2010) Morpho-histological study of direct somatic embryogenesis in endangered species *Fritillaria meleagris*. *Biologia Plantarum* **54 (3)**, 592-596

Sun D (2010) The metabolism of carbohydrates in *Fritillaria ussuriensis* Maxim. during different stages of tissue culture. *Medicinal Plant* **1 (3)**, 4-8

Sun CS, Chu CC, Wang CC (1977) Callus formation and organ regeneration in the tissue culture of *Fritillaria thunbergii* Miq. *Acta Botanica Sinica* **19**, 161-162*

Sun CS, Wang DY (1991) Fritillaries (Fritillary): *In vitro* culture and the regeneration of plants. In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry: Medicinal and Aromatic Plants* III (1st Edn, Vol XV), Springer, Berlin, Germany, pp 258-269

Sun D, Piao XC, Lian JS, Lian ML (2008) Study on several factors affecting callus growth of *Fritillaria ussuriensis* Maxim. *Northern Horticulture* **4**, 218-220

Tang K, Wang X, Sun X, Deng Z (2006) Cold: A double-edged sword to plants: Stress in plant growth and development: Mechanism and pathways. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol III), Global Science Books, Isleworth, UK, pp 102-107

Tang W, Yinggen Y, Gui Y, Guo C, Wu J (1996) Influence of culture condition on callus differentiation of *Fritillaria ussuriensis* M. *Biotechnology* **4**, 11-14

Tingyao J, Huibin D, Lunshan W (1999) Study on secondary metabolites in the tissue culture of *Fritillaria pallidiflorae* Schrenk. *Acta Botanica Boreali-occidentalia sinica* **1**, 130-134*

Tsoi PY, Woo HS, Wong MS, Chen SL, Fong WF, Xiao PG, Yang MS (2003) Genotyping and species identification of *Fritillaria* by DNA chips. *Acta Pharmaceutica Sinica* **38 (3)**, 185-190*

Ulug BV, Korkut AB, Sisman EE, Vuz M (2010) Research on propagation methods of Persian lily bulbs (*Fritillaria persica* Linn) with various vegetative techniques. *Pakistan Journal of Botany* **42**, 2785-2792

Van Leeuwen PJ, Dop AJ (1990) Effects of storage, cooling and greenhouse condition on *Anemone blanda, Fritillaria meleagris* and *Oxsalis adenophylla* for use as potplant. *Acta Horticulturae* **266**, 101-107

Van Leeuwen PJ, Trompert JPT, Van Der Weijden JA (2002) The forcing of *Fritillaria imperialis* L. *Acta Horticulturae* **570**, 165-169

Wang HP, An L, Chen Y, Hou Q, Huang R (2009) Regeneration induction of *Fritillaria przewalskii* bulbs with different phytohormones composition *in vitro*. *Journal of Gansu Agricultural University* **44 (3)**, 50-52*

Wang L, Wang Y, Yang H (1987) Study on chromosome unstability in tissue culture of *Fritillaria pallidiflorae* under different hormones. *Acta Botanica-occidentalia Sinica* **4**, 226-234*

Wang L, Ding H, Wang H, Kang W (1990) Study of chromosome variation and differentiation frequency in callus subcultures of *Fritilariae pallidiflorae*. *Acta Botanica Boreali-occidentalia Sinica* **1**, 54-60*

Wang LS, Yang HM, Wang YF, Kung WJ (1989) Somatic embryogenesis from tissue culture of *Fritillaria pallidiflora* and cytohistological observation. *Acta Botanica Boreali-occidentalia Sinica* **9 (2)**, 76-81*

Wang S, Gao W, Chen H, Xiao P (2005a) New starches from *Fritillaria* species medicinal plants. *Carbohydrate Polymers* **61**, 111-114

Wang S, Gao W, Yu J, Xiao P (2005b) Identification of *Fritillaria* herbal drugs by X-ray diffraction of starch grains. *China Journal of Chinese Materia Medica* **30 (11)**, 805-807

Wang S, Yu J, Gao W (2005c) Use of X-ray diffractometry (XRD) for identi-

fication of *Fritillaria* according to geographical origin. *American Journal of Biochemistry and Biotechnology* **1 (4)**, 199-203

Wang S, Gao W, Jia W, Xiao P (2006) Crystallography, morphology and thermal properties of starches from four different medicinal plants of *Fritillaria* species. *Food Chemistry* **96**, 591-596

Wang YH, Dai Y, He ZS, Sun YX, Yan SJ, Xu SJ, Wang XR (2010a) The effects of *in vitro* culture conditions on regeneration of *Fritillaria cirrhosa*. *Zhong Yao Cai* **33 (6)**, 854-856*

Wang YH, Dai Y, Yan SJ, Sun YX, He ZS, Xu SJ, Wang XR (2010b) Study on fast proliferation conditions of *Fritillaria cirrhosa* D. Don callus. *Southwest China Journal of Agricultural Sciences* **23 (2)**, 528-531*

Wang YH, He ZS, Sun YH, Ma LL, Liu YL, Lin KX (2011) Study on the production of alkaloid by cell mass suspension culture of *Fritillaria cirrhosa*. *Zhong Yao Cai* **34 (2)**, 183-186*

Witomska M, Lukaszewska A (1997) Bulblet regeneration *in vitro* from different explants of *Fritillaria imperialis*. *Acta Horticulturae* **430**, 331-338

Witomska M, Wilk T, Lukaszewska A (1998) Effect of sterilization method on infection level and regeneration *in vitro* of explants from bulbs of *Fritillaria imperialis* L. *Scientific Papers of the Institute of Pomology and Floriculture* **5**, 121-130 (in Polish with abstract in English)

Witomska M (2000) The effect of the bulb storage temperature on the levels of soluble sugars, ABA and cytokinins and the *in vitro* regeneration of *Fritillaria imperialis* L. *Problem Papers Progress of Agricultural Sciences* **473**, 335-341 (in Polish with abstract in English)

Wu J, Tang W (1992a) Studies on tissue culture of medicinal plant (*Fritillaria ussuriensis* Maxim) 1. Induction of callus, organogenesis and cytological observation. *Journal of Northeast Agricultural University* **2**, 188-195*

Wu J, Tang W (1992b) Studies on tissue culture of medicinal plant (*Fritillaria ussuriensis* Maxim) 2. Somatic embryogenesis and cytological observation. *Journal of Northeast Agricultural University* **4**, 48-54*

Xiao PG, Jiang Y, Li P, Luo YB, Liu Y (2007) The botanical origin and pharmacophylogenetic treatment of Chinese materia medica Beimu. *Acta Phytotaxonomica Sinica* **45 (4)**, 473-487*

Xu D, Gao S, Cai ZZ (1992) Study on medium selection and simplification in bulb culture of *Fritillaria unibracteata*. *Journal of China Pharmaceutical University* **23 (5)**, 304-306*

Xu Y, Zhu S (1998) Comparison of embryoid induction condition of Siberian fritillary and ussuri fritillary. *Journal of Shaanxi Normal University (Natural Science Edition)* **26 (2)**, 123-124*

Xue JP, Zhang AM, Geng ML, Ma L (2008) Study on bulblet induction of *Fritillaria anhuiensis in vitro*. *China Journal of Chinese Materia Medica* **33 (22)**, 2603-2606*

Yamazaki H, Nishijima T, Koshioka M, Miura H (2002) Gibberellins do not act against abscisis acid in the regulation of bulb dormancy of *Allium wakegi* Araki. *Plant Growth Regulation* **36**, 223-229

Yang SR, Tai CD, Chen CC, Tsay HS (2001) Effect of plant growth regulators and status of the medium on induction and proliferation of protocorm-like bodies and bulblets from bulbscale culture of *Fritillaria hupehensis* (Hsiao et K.C.Hsia). *Journal of the Agricultural Association of China* **2 (5)**, 414-422*

Yang Y, Jiang H, Fu HL, Lan LQ (2008) Determination and histochemical localization of alkaloids content in wild and tissue cultured *Fritillaria cirrhosa* D. Don. *Journal of Sichuan University* **45 (1)**, 209-213*

Yu J, Wei J, Chen S, Dai Y, Yang C (2008) Dormancy and germination characteristics of *Fritillaria cirrhosa* seed. *Chinese Traditional and Herbal Drugs* **7**, 1081-1084*

Yu KJ, Paek KJ, Seong NS, Choi IS, Cho JT (1994) Establishment of *in vitro* culture and effect of chilling treatment of mother bulb on bulblet formation in *Fritillaria thunbergii* Miq. *Korean Journal of Medicinal Crop Science* **2 (3)**, 211-218 (in Korean with abstract in English)

Yu SC, Xiao PG (1992) The existence of isosteroidal alkaloids in *Fritillaria* L. (*Liliaceae*) and its taxonomical significance. *Acta Phytotaxonomica Sinica* **30**, 450-459*

Yuan Y, Lin HH, Wang WJ, Li P (2005) Callus induction and plant regeneration from bulbs of *Fritillaria thunbergii*. *Journal of Southwest Jiaotong University* **40**, 281-284*

Zhao GF, Cao Y, Wu Y, Fan F, Zhou LJ, Yang WH (1983) Callus induction and organ regeneration in tissue culture of *Fritillaria ussuriensis* Maxim. *Chinese Bulletin of Botany* **1**, 40-41*

Zhang MP, Wang Y, Wei HL, Tag JL, Pang H, Li XG (2003) Study on secondary metabolism of *Fritillaria ussuriensis* Maxim. *Journal of Jilin Agricultural University* **25 (2)**, 171-174*

Zhang SW, Lei ZQ, Liu H, Cao L, Wu ZG (2007) Studies on tissue culture and selection of high-yield cell line of *Fritillaria monatha* Migo. *Acta Agriculturae Universitatis Jiangxiensis* **29 (1)**, 152-155*

Zhou BJ (1983) A study on *in vitro* culture of bulb of *Fritillaria ussuriensis* Maxim. *Plant Physiology Communication* **1**, 30*

Zhou SB, Wang Y, Li JH, Wang CJ, Yu BQ (2007) Morphology of leaf epidermis of *Fritillaria* in Anhui province. *China Journal of Chinese Materia Medica* **32 (2)**, 105-109*

Zhu D, Jiang Y, Chen TG (1992) Research on chemical compounds and primary pharmaceutical action of cultured bulb of *Fritillaria unibracteata*. *Journal of China Pharmaceutical University* **23 (2)**, 118-121*

Zhu SY, Hu ZH, Yu WQ (1980) Study on the annual periodicity of growth and development of *Fritillaria pallidiflora* Schrenk. *Journal of Integrative Plant Biology* **22 (1)**, 22-26*

Zhu Y, Chen F, Zhang A, Sheng W, Song Y (2011) Study on preservation of *Fritillaria anhuiensis* by vitrification *in vitro*. *Zhongguo Zhong Yao Za Zhi* **36 (18)**, 2462-2464*

Ziv M, Lilien-Kipnis H (2000) Bud regeneration from inflorescence explants for rapid propagation of geophytes *in vitro*. *Plant Cell Reports* **19**, 845-850