Scientific Bulletin. Series F. Biotechnologies, Vol. XX, 2016
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ACCELERATED MICROPROPAGATION OF ENDEMIC *FRITILLARIA AUREA* SCHOTT

Suleyman KIZIL[1*], Tahsin SOGUT[1], Ugur SESIZ[2], Khalid Mahmood KHAWAR[3]

[1]Dicle University, Faculty of Agriculture, Department of Field Crops, Ziraat Fakultesi, 21280 Diyarbakir,Turkey,
[2] SirnakUniversity, Faculty of Agriculture, Department of Field Crops, Idil, 73300Sirnak, Turkey
[3]Ankara University, Faculty of Agriculture, Department of Field Crops, Kecioren Fatih, 06110 Ankara, Turkey

*Corresponding author: suleymankizil@gmail.com

Abstract

Fritillaria aurea Schott, an *Iran-Turan element, is a rare alpine geophyte with spotted brown yellow flowers. The plant is endemic to Turkey with dispersion in the South-Eastern Anatolia region. This study reports in vitro culture of Fritillaria aurea previously collected from the provinces of Adiyaman and Malatya in the South-Eastern Anatolia Region and presently cultured at the Ornamental Plants Garden Collection of Faculty of Agriculture, Dicle University, Diyarbakir, Turkey. Bulblets were cultured on MS medium containing different concentrations of TDZ, TDZ andNAA. Callus induction followed by bulb formation was noted on bulblets. The bulblets obtained these were cultured on MS medium containing 40 or80 g/l sucrose to increase bulb diameter. The in vitro regenerated bulblets were rooted on MS medium containing different concentrations of IBA (0.25, 0.50, 0.75 and 1.0 mg/l) supplemented with 30 g/l sucrose. A significant increase in number of bulblets and bulb diameter was noted on F. aurea Schott bulbs.*

Key words: *Bulblets, in vitro, multiplication, sucrose.*

INTRODUCTION

The *Liliaceae* contains approximately 280 genera and 4000 species. There are approximately 430 Liliaceous species in Turkey and there are 49 taxa of *Fritillaria* that grows in wild in Turkey with endemism ratio is 36.53% in *Fritillaria* (Teksen at al., 2011).

The *Fritillaria aurea* Schott is a rare alpine geophyte with spotted brown yellow flowers. The plant is grown at rocky and high places average between 1600-3000 m.

It is an Iran-Turan element and has rich potential for use in alpine ornamental landscaping. The plant is endemic to Turkey and disperses in Mersin, Kayseri, Malatya, Nigde, Sivas and Adiyaman provinces (Bakis et al., 2011).

Although Turkey is signatory to CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora, also known as the Washington Convention), wild populations of the plant are getting reduced with the passage of time largely due to illegal collections for local cut flower industry and traditional medicinal systems, fast urbanisation and very limited measures to conserve local germplasm.

Fritillaria extracts are widely used in traditional Turkish, Chinese, and Indian medicinal systemsto treat cough inflammation and like. *Fritillarias* are widely used to treat cough, lung disorders, lumps beneath the skin and cancers in traditional folk medicines of Turkey.

The rate of natural vegetative multiplication is generally very low in geophytes including *F. aurea* Schott. It takes 5 – 6 years to produce a plant capable of flowering from seed under ideal conditions in the wild. Plant tissue-culture technology is playing an increasingly important role in basic and applied studies, including crop improvement (Brown and Thorpe, 1995). Thus, the techniques like plant tissue culture, could offer an attractive alternative for increasing propagation efficiency especially for commercial production as has been used in many other geophytes and bulbous plants (Bach and Sochacki, 2013).

Fritillaria aurea Schott has huge potential for their use in parks, gardens as indoor and outdoor ornamental plant. Multiplication of plant through plant tissue culture offers many advantages when it is compared to traditional propagation techniques formas propagation that allows accelerated rates of propagation for higher yield of plants in shorter time. Plant tissue also offers a very beneficial pathway for rapid production of clonal elites; where natural production rates of the plants are very slow.

Successful regeneration of plants via organogenesis from vertically sliced bulb halves of this plant has never been reported. This paper reports a protocol for bulblet multiplication leading to acclimatization that also included optimisation of the effects of different concentrations of sucrose and IBA on bulblet growth and root formation. The objectives of the present work are to establish an efficient *in vitro* propagation method for *Fritillaria aurea* Schott as an alternative to the conventional approach using vertically sliced bulbs through *in vitro* manipulation. All stages of organogenesis were identified and complete plantlets were formed and finally successfully acclimatized. Comparison of morphological characteristics between *in vivo* and *in vitro* grown plants was also made.

MATERIALS AND METHODS

Plant materials and experiments

The study made use of *in vitro* regenerated bulblets (Kizil et al., 2013). *Fritillaria aurea* Schott (Figure 1) bulblets were obtained from the previous experiment using MS medium containing0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10mg/l NAA under *in vitro* conditions (primary medium).These bulblets were induced on light yellow coloured compact mass calluses and after four months of culture.

The bulblets achieved desired gain in bulblet diameter they were rooted on MS basal medium containing 3×KNO_3 and supplemented with 0.25, 0.50, 0.75 or1.0mg/l IBA, 30.0 g/l sucrose (w/v) and solidified with 6.2 g/l agar (w/v) in Magenta GA^7 vessels for rooting(secondary medium medium).These were cultured on modified MS medium(170 mg/l potassium phosphate, 1900 mg/l potassium nitrate, 100mg/l inositol, 0.5mg/l nicotinic acid, 0.5mg/lpyridoxine, 0.1mg/l thiamine and 2.0mg/l glycine were added to MS basal medium) containing sucrose (40or80 g/l) solidified with 6.2 g/l agarat 24° ± 1°C in sterile Magenta GA^7 vesselsfor28 weeks(tertiary medium).The pH of all cultures medium was adjusted to 5.6 - 5.8 with 0.1 M KOH or 0.1 M HCl before autoclaving at 121ºC, 117.7 kPa for 20 min.

Figure 1. *Fritillaria aurea* Schott growing at its natural habitat

Hardening and acclimatization

The well-developed bulbs were washed thoroughly in running tap water transferred to plastic pots containing sterilised peat moss under greenhouse under controlled conditions of temperature (24º± 1ºC) and light 3000 lux (16/8 h photoperiod) conditions. Potted plantlets were covered with transparent plastic bags to ensure high humidity and watered everyday with water for 15 days to acclimatize them.

All cultures unless otherwise mentioned separately, were grown in Fitotron growth chamber (FitotronSGC 120; Epinal Way, Loughborough, UK) with 16 h of cool white fluorescent light (Philips lamps TLD 36 W/54, Hungary) at a photon flux density of 35 µmol/ m^2/sper day.

All experiments made use of60 explants equally divided into 10 replications. Statistical analysis was performed using IBM SPSS 22 program for windows by comparing means for One Way ANOVA. All values expressed in percentage were arcsine transformed before statistical analysis (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSIONS

Results

The bulblets were induced on callus proliferated by MS medium containing 0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10mg/l NAA (primary medium–Figure 2, Figure 3 a).

These bulblets as shown in the materials and methods did not induce roots, therefore there were rooted on MS medium containing 0.25, 0.50, 0.75 or 1.0 mg/l IBA for rooting (secondary medium–Figure3 b). These applications helped to increase rooting percentage, number of roots per bulblet, root length, number of shoots per bulblet, shoot length, bulblet diameter and number of bulblets per explant bulb used as explant variably. Maximum rooting percentage (100%), and number of roots per bulblet(6.35) were note on 0.50 mg/l IBA. Maximum root length (2.73 cm) was noted on 1 mg/l IBA (Table 1).Mean number of roots of on bulblets treated with0.25mg/l IBA were higher compared toother concentrations of IBA. Maximum number of shoots per bulblet (6.44) and number of bulblets per stalk bulbs(11.90) were noted on 0.25 mg/l IBA with significant differences among other concentrations of IBA.

Figure 2.*Fritillaria aurea* Schott plants in *in vitro* bulblets formation on TDZ (Thidiazuron) medium

IBA did not induce meaningful variations in bulblet diameter. The bulblets diameter ranged from 0.58 to 0.64 cm. Maximum shoot length (5.80 cm) and bulblet diameter (0.64 cm) was noted on0.75 mg/l IBA. However, all concentrations of IBA also induced number of bulblets on mother or stalk bulbs used as explant. All concentrations of IBA affected and transformed vegetative bulblet tissues to generative tissues that besides helping in root induction also induced variable number of axillary bulblets on all concentrations of IBA used in the study.

It was thought that it is an important situation for *F. Aurea* Schott induction of productive bulblets under *in vitro* conditions. These IBA induced bulblets were cultured on MS medium containing 40 or80 g/l sucrose to provide increase in their diameter (tertiary medium). MS medium containing 40 g/l sucrose induced higher values for root induction percentage and number of roots per explants, root and shoot length, bulblet diameter and number of bulblets *per* explant compared to the bulblets cultured on 80 g/l sucrose.

Number of roots on modified MS containing 40 g/l application was 1.44, root length as 0.73 cm, shoot length 2.20 cm, bulblet diameter 0.64 cm and number of bulblet as 1.58. Increasing sucrose amount in the medium had affected negatively and investigated characteristics gave low values (Table 2; Figure 3 c, d).

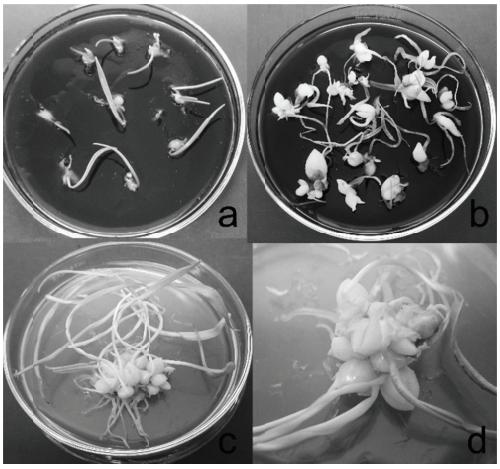

Figure 3. Micropropagation of *Fritillaria aurea* Schott (a) TDZ (Thidiazuron) regenerated bulblets (b) bulblets transferred to IBA (Indole-3-butyric acid) containing medium for rooting (c, d) axiallary bulblet regeneration on stalk bulbs

These bulbs were transferred to pots containing peat and watered daily with 0.5 ml water for a period of 4 weeks until the established and showed profuse growth of green leaves. All of them were physiologically normal and did not show any sign of abnormal growth during culture in the greenhouse; where their pots

were kept under controlled conditions of temperature and humidity.

Table 1. Effects of different IBA (Indole-3-butyric acid) concentrations on some investigated characteristics of *Fritillaria aurea* Schott

IBA (mg/l)	Rooting rate (%)	Number of roots per bulblet	Root length (cm)	Number of shoot per bulblet	Shoot length (cm)	Bulblet diameter (cm)	Number of bulblets per explant
0.25	76.66	4.73	2.60	6.44	1.41 b	0.59	11.90 a
0.50	100.00	6.35	1.95	5.05	5.30 a	0.58	4.65 b
0.75	80.00	5.58	2.65	5.00	5.80 a	0.64	5.45 b
1.00	95.00	4.78	2.73	3.78	4.05 a	0.62	3.55 b

Means within a column followed by the same letter are not significantly different according LSD test at $p \leq 0.05$.

Table 2. Effects of different sucrose concentrations on growth of *Fritillaria aurea* Schott bulbs obtained from different IBA (Indole-3-butyric acid) concentrations

Sucrose (g/l)	Rooting rate (%)	Number of roots per explant	Root length (cm)	Number of shoot per explant	Shoot length (cm)	Bulblet diameter (cm)	Number of bulblets per explant
40	52 a	1.44 a	0.73 a	2.90 a	2.20	0.64 a	1.58 a
80	40 b	0.84 b	0.32 b	1.04 b	1.16	0.49 b	0.84 b

Means within a column followed by the same letter are not significantly different according to LSD test at $p \leq 0.05$.

Discussions

The present study confirmed regeneration of bulblets induced on TDZ and NAA concentrations in agreement with previous studies.TDZ has been shown to induce callus formation in a variety of plant culture systems due to very high rate of cell proliferation. TDZ induced a 30-fold increase in the growth of callus cultures over other plant growth regulators (Capelle et al., 1983) with a relatively high intrinsic activity in agreement with present studies, previous studies also confirm thatTDZ in combination with 2,4-D,IBA (Passey et al., 2003;Yonghua et al., 2005) or NAA (Erisen et al.,2011) were effective to regenerate plantlets from *Fragaria* leaves and *Astragalus* species respectively. However, present studies indicate that the bulblets that were induced on callus proliferated by MS medium containing 0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10 mg/l NAA, they failed to increase in diameter. It is assumed that this could be due to the influence of TDZ based a relatively high level of accumulation of minerals or other metabolites in callus tissues that induced stress causing hinderances in the development of tissues in agreement with Murch et al. (1997). Resultantly, to overcome this physiological stress, the induced callus tissues might have modified their metabolic processes that ended up in hinderances to growth of newly regenerated bulblets.

Another explanation could be TDZ induced bulblets failed to elongate due to high carry over effect or partial toxic activity of TDZ in agreement with Huetteman and Preece (1993).The problem was overcome by transfer of bulblets a secondary medium containing IBA lacking TDZ and NAA that created a different balance and improved the growth of bulbs transforming the tissues from vegetative to generative phase. Use of secondary media for shoot proliferation has been reported in many plants like *Malus* (Fasolo et al., 1989), *Pyrus* (Singhaand Bhatia, 1988), *Populus* (Russell and McCown, 1988), and *Rhododendron* (Preece and Imel, 1991). All researchers used primary media to maximize shoot proliferation, and secondary media for their elongation and growth. Some researchers also used alternatively, chilling treatments can be used for shoot elongation. Briggs et al. (1988a, 1988b) also used chilling treatments on TDZ induced *in vitro* grown *Rhododendron* sp. (deciduous azaleas) shoots to 3°C for 2 months; which elongated rapidly once they were shifted to light and warm growing conditions. The results of the present study showed that that IBA acted as recovered to these effects and culture of individual bulblets on it had positive effects on both bulblet regeneration, tissue maturity and production of axillary bulblets.

TDZ induced bulblets changed their response on IBA containing secondary regeneration medium where axillary bulblets and roots were noted that retreated directly from the mother or stalk bulblets produced *in vitro* and were very noticeable after 4 to 5 weeks of culture.

It seemed as if IBA ameliorated the negative impacts of TDZ and all bulblets rooted variably. The supply of IBA increased the root length, root fresh weight, plant height and shoot fresh weight on increased IBA concentrations (0.50 to 2.00 mg/l). Maximum rooting was noted on 0.50 mg/l IBA. Increased TDZ concentrations increased leaf number, plant height and shoot fresh weight. No consistent differences for IBA effects were detected between shoot characters in *in vitro* derived plantlets. Regardless of IBA concentrations, bulblets in 0.50 mg/lIBA produced the best rooting system with vigorous shoot characteristics when they were treated with two concentrations of sucrose. However, 40 g/l sucrose seemed more favourable for development and growth of plants. This affected survival rate of acclimatized plants in as well.

In the present study, for the first time, a protocol for plantlet regeneration on MS medium containing high TDZ concentrations combined with IBA was developed. Regeneration ability of *Fritillaria* species depends on genotype of plant, growing conditions and *in vitro* medium and conditions. Kukulczanka et al. (1989) reported that they had obtained adventives bulblets by using bulb scale and full bulb. The highest regeneration rate was obtained from multiplication of cytokinin and auxins in their study. Contrarily this study showed overcoming negative effects of TDZ and bulblet regeneration using IBA and sucrose. The findings are new and have never been reported for Fritillarias or any bulbous plant species.

Rahimi et al. (2014) reported that plant tissue culture is a technique that has ability of production of a large number of high quality plants. In *Fritillaria imperialis*, the number of regenerated shoots was the highest on MS medium supplemented with 0.5 mg/l TDZ and 30 g/l sucrose; the number of roots was the highest on MS medium supplemented with 0.2 mg/l NAA and 30 g/l sucrose; the *in vitro* bulb diameter is largest. Marija et al. (2011), reported that somatic embryogenesis and whole plant regeneration were achieved from mature zygotic embryos of *Fritillaria meleagris*. They reported also embryogenic callus was derived from mature zygotic embryos after 4 weeks on TDZ containing medium. Somatic embryos at the early stages of development arose from the surface of the embryogenic callus. They had multiplication of somatic embryos, formation of bulblets and shoot development observed on the same medium. The results reported in this study are not in agreement with previous study and have edge over the previous studies. It reports recovery of TDZ regenerated bulblets on IBA and sucrose containing medium. Moreover, the regenerated bulblets were physiologically normal and did not show any abnormality on growth when they were cultured and transferred to pots for growth.

CONCLUSIONS

In conclusion, the present study underlines the importance of primary, secondary and tertiary medium for bulblet regeneration rooting and increasing bulblet diameter of *F. aurea* Schott by organogenesis.

The results also indicate partial cell specific inhibition due to TDZ induced regeneration of under *in vitro* conditions.

The results of the study are novel and could be effectively used during conservation strategies and commercial multiplication studies.

ACKNOWLEDGEMENTS

This work was supported by a grant (Project number: TUBITAK 110 O 703) from the Scientific and Technical Research Council of Turkey (TUBITAK).

REFERENCES

Bach, A., Sochacki, D. 2013. Ornamental Geophytes from Basic Science to Sustainable Production; Phsiology and Menagement Systems. Kamenetsky, R and Okubo, H (Eds.), CRC Press, Boca Raton, http//:www.crcpress.com.

Bakis, Y., Babac, M.T., Uslu, E. 2011. “Updates and improvements of Turkish Plants Data Service (TÜBİVES)" In Health Informatics and Bioinformatics (HIBIT), 2011 6th International Symposium on (pp. 136-140). IEEE.

Briggs, B.A., McCulloch, S.M., Edick, L.A. 1988a.Micropropagation of azaleas using thidiazuron. Acta Hort. 226: 205-208.

Briggs, B.A., McCulloch, S.M., Edick, L.A. 1988b.Micropropagation of azaleas using thidiazuron. Acta Hort. 227: 330-333.

Brown, D.C.W., Thorpe, T.A. 1995. Crop improvement through tissue culture. World Journal of Microbiology &Biotechnology, 11: 409-415.

Capelle, S.C., Mok, D.W.S., Kirchner, S.C., Mok, M.C. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(Y2- isopentyl) [8-14c] adenosine in callus tissues of *Phaseolulunatus* L. Plant Physiol. 73: 796-802.

Erisen, S., Atalay, E., Yorgancılar, M. 2011. The effect of thidiazuron on the *in vitro* shoot development of endemic *Astragalus cariensis* in Turkey. Turk J Bot. 35: 521-526.

Fasolo, F., Zimmerman, R.H., Fordham, I. 1989. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16: 75-87.

Huetteman, C.A., Preece, J.E. 1993. Thidiazuron-A potent cytokinin for woody plant-tissue culture. Plant Cell Tissue Org. Cult. 33(2): 105-119.

Kizil, S., Sesiz, U., Khawar, K.M., Arslan, N. 2013. Studies on *in vitro* Micropropagation of *Fritillaria aurea* Schott. V. Ornamental Plants Congress, 06-09 May 2013 Yalova, Turkey, Volume II, 602-605.

Kukulczanka, K., Kromer, K., Czastka, B. 1989. Propagation of *Fritillaria meleagris* L. through tissue culture. ActaHortic. 251: 147–153.

Marija, P., Angelina, S., Sladana, J., Milana, T. 2011. Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). Afr.J.Biotechnol. 10(72): 16181-16188.

Murch, S.J., KrishnaRaj, S., Saxena, P.K. 1997. Thidiazuron-induced regeneration:A potential stress response. Plant Physiol. 114(3): 177-177.

Passey, A.J., Barrett, K.J., James, D.J. 2003. Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria* x *ananassa* Duch.) using a range of explant types. Plant Cell Replication, 21:397-401.

Preece, J.E.,Imel, M.R.1991. Plant regeneration from leaf explants of Rhododendron "P.J~M. Hybrids'. Scientia Hort. 48 (1-2): 159-I70.

Rahimi, M., Daneshvar, M.H., Heidari, M. 2014. Propagation and bulb formation of *Fritillaria*(*Fritillaria imperialis* L.) Via *in vitro* culture. International Journal of Plant, Animal and Environmental Sciences, 4: 707-710.

Russell, J.A.,McCown, B.H. 1988. Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Rep. 7:59-62.

Singha, S., Bhatia, S.K. 1988. Shoot proliferation of pear cultivars on medium containing thidiazuron and benzylaminopurine. Hort. Sci. 23: 803.

Snedecor, G.W., Cochran, W.G. 1967. Statistical methods. 593 p. The Iowa State University Press, Ames, Iowa, USA.

Teksen, M., Aytac Z. 2011. The revision of the genus *Fritillaria*L. (Liliaceae) in the Mediterranean region (Turkey)., Turk. J. Bot. 35: 447-478.

Yonghua, Q., Shanglong, Z., Asghar, S., Lingxiao, Z., Qiaoping, Q., Kunsong, C., Changjie, X. 2005. Regeneration mechanism of Toyonoka strawberry under different color plastic films. Plant Sci. 168:1409-1424.