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HAPLOID INDUCTION VIA IN VITRO GYNOGENESIS IN PERSIAN SHALLOT (*ALLIUM HIRTIFOLIUM*)

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ABSTRACT

Haploid induction using in vitro cultures of unpollinated flowers has been recognized as an important tool to produce homozygous plants for genetic studies and breeding programs. In this study the potential of gynogenic haploid induction in four ecotypes of *Allium hirtifolium* under different combinations of benzylaminopurine (BAP) with 2,4-dichlorophenoxyacetic acid (2,4-D), or α-naphthaleneacetic acid (NAA) was investigated. Unpollinated flower buds were excised from an umbel 5 to 3 days before anthesis, and cultured onto B5 medium containing 7.5% sucrose and 2 $mg \cdot dm^{-3}$ BAP with auxin. The experiments revealed that NAA increased the percentage of gynogenesis induction and number of gynogenic embryos per flower in all ecotypes. Somatic organogenesis from basal callus or other floral parts was most effective on the media containing 2,4-D. Plants obtained by gynogenesis were haploid in 70–77% and plants from somatic tissue were mostly diploid.

Keywords: flower culture, embryogenesis, regeneration from somatic tissues, mixoploids

INTRODUCTION

Allium hirtifolium Boiss. commonly known as Persian shallot is an important wild medicinal plant distributed from North West to central and South West of Iran. For many years, fresh and dry bulbs of *A. hirtifolium* are used in herbal medicine to treat rheumatic, inflammatory, arthritis, diarrhea, and stomach pains (Asili et al. 2010). The antimicrobial, antifungal, antiparasitic and antioxidant activities of the bulb extract have been reported (Souri et al. 2008; Taran & Izaddoost 2010). Persian shallot has recently gained significant attention because it produces high amounts of many useful compounds, such as allicin, phenolic antioxidants, and ascorbic acid (Asili et al. 2010; Ghasemi Pirbalouti et al. 2015).

Conventionally, *A. hirtifolium* propagates through bulbs and seeds, but these two methods are not commercially efficient due to low growth rate of bulbs and deep dormancy of seeds (Dashti et al. 2012; Ebrahimi et al. 2014). The breeding process of Persian shallot due to low propagation rate is slow, and for that reason breeding on *A. hirtifolium* was rarely undertaken. The exception was determination of genetic diversity among its natural populations (Ebrahimi et al. 2009; Asili et al. 2010). Therefore, the preparation of suitable plant material for the improvement of the traditional *A. hirtifolium* breeding programs is necessary. In recent years, one of the most significant biotechnological advances in plant breeding has been the development of in vitro techniques to produce haploid plants that can be used to generate homozygous lines by induced chromosome doubling (Alan et al. 2004; Ibrahim et al. 2016). Pure lines can provide direct establishment of new cultivars in autogamous species or could be used to produce high-yielding hybrids mainly in allogamous species (Veilluex 1994). Several references showed that haploid induction in genus *Allium* via gynogenesis seems to be an appropriate method to produce haploid plants and has a great potential for breeding (Bohanec & Jakše 1999; Alan et al. 2003; Fayos et al. 2015). Successful haploid induction has been reported in different *Allium* species through the unpollinated flower culture (Alan et al. 2004; Sulistyaningsih et al. 2006;

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Ebrahimi & Zamani 2009; Fayos et al. 2015), while no success has been reported using anther culture (Keller 1990; Sulistyaningsih et al. 2006).

Haploid induction is influenced by genotype, culture conditions, media components, and growth regulators (Shalaby 2007). To achieve successful rates of gynogenesis, several factors including genotype and growth conditions of donor plants, mineral contents of culture medium, and plant growth regulators need to be considered (Bohanec 2009). The success of direct embryo production particularly in gynogenic embryo induction in ovule, ovary, or flower bud culture depends upon the combination of cytokinin and auxin. The combination of 6-benzylaminopurine (BAP) with auxins has a favorable effect on gynogenesis of alliums (Campion et al. 1992). Also, the choice of suitable genotype used as donor plant has a great effect on gynogenic embryo induction (Ibrahim et al. 2016).

The objective of this study was to develop an effective medium for gynogenic embryo induction and haploid plants regeneration from ecotypes of *A. hirtifolium* using flower bud culture.

MATERIALS AND METHODS

Plant material

The bulbs of *A. hirtifolium* Boiss. were collected from natural habitats in four regions of Iran: Arak, Lorestan, Sanandaj, and Yasoj. The bulbs were planted in the experimental field at University of Tabriz, Iran, in November 2014. All bulbs flowered during April–May. In general, plants formed one scape with umbel which had 100–200 flower buds. Unopened flowers collected from an umbel 5–3 days before anthesis were used for inoculations. The collected flowers were sterilized with 70% ethanol for 10 s and then with 2% hypochlorite sodium solution for 5 min and finally rinsed three times with sterile water.

Effect of auxin on gynogenesis induction

The effect of two auxins – 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthaleneacetic acid (NAA), at the concentrations 1 and 2 $mg \cdot dm^{-3}$, on gynogenic embryo induction was investigated. The B5 medium (Gamborg et al. 1968) containing 2 $mg \cdot dm^{-3}$ BAP, 7.5% (w/v) sucrose, and solidified with 0.8% agar (Agar-agar) was used. All components were purchased from Merck Company (Germany). The pH of the media was adjusted to 5.7 before autoclaving for 20 min at 121 °C. Flowers were plated on medium in jars, eight flowers each. Cultures were maintained at a temperature of 25 ± 1 °C under a 16/8 h light regime of 40 $\mu mole \cdot m^{-2} \cdot s^{-1}$ provided with white fluorescent tubes. Subsequently, subculturing was done on the same media every 4 weeks. The following parameters were recorded: the percent of gynogenesis induction in cultured flowers, the number of gynogenic embryos per flower, the percent of embryos which developed into plantlets, the percent of flowers that formed callus, and the number of plantlets that were formed outside the ovaries. For shoot induction, the obtained calli were sub-cultured onto MS medium (Murashige & Skoog 1962) containing 1.5 $mg \cdot dm^{-3}$ BAP and 0.5 $mg \cdot dm^{-3}$ NAA with 3% sucrose and 0.8% agar.

Statistical analysis

The experiments were designed on the base of complete randomization, with four replications and 40 explants in each replication. Results were analyzed statistically using the Statistical Analysis Program (SPSS ver. 16.0). The mean values were calculated and compared by Duncan's multiple range tests ($p < 0.05$).

Cytogenetic analysis

The gynogenetic plantlets, plantlets directly regenerated from outside of ovaries, and callus-derived plantlets were transplanted for rooting onto free hormonal MS medium containing 3% sucrose and 0.8% agar. Root tips of newly developed roots, taken from each regenerated plantlet were pretreated with alpha-bromo-naphthalene at 3 °C for 8 h and fixed in a mixture of acetic acid and ethyl alcohol (1:3 v/v) for 3–4 days. After hydrolysis in 1 N hydrochloric acid at 60 °C for 8 min, they were stained with orcein 2% and squashed in 45% acetic acid. The best metaphase plates were photographed under a Nikon microscope (E 400) equipped with a Nikon digital camera (DXM 1200). The mean of 15 metaphase plates was analyzed per plant.

RESULTS AND DISCUSSION

The production of pure lines in different crops especially in open-pollinated plants requires both time and adequate facilities. Pure lines can be produced from haploid plants in a short time using in vitro techniques and therefore save several years compared with conventional plant breeding program (Wang et al. 2014). In this study, we investigated the potential of production of haploid plants of *A. hirtifolium* via in vitro gynogenesis and the optimum combination of auxins for gynogenesis induction in four ecotypes of this species.

Analysis of variance showed significant differences resulting from type and concentration of auxin and also from ecotype for the percent of gynogenic induction in cultured flowers, number of gynogenic embryos, the percent of callus induction (Table 1) and for directly produced somatic embryos per flower (Fig. 1). Interaction effects of treatments were significant for all of the studied parameters with the exception of the directly induced somatic embryo numbers.

Cultured flower buds opened 1 week after culture beginning. After 20 days from the start of culture, ovaries enlarged. According to Campion et al. (1992), gynogenic organogenesis from flower culture needed around 65–70 days to develop and become visible. Grzebelus and Adamus (2004) reported that gynogenic onion embryos were emerged from ovaries from 8th to 30th week of in vitro culture of unpollinated flower buds. In this study, gynogenetic embryos that originated directly from inside of ovules emerged after 56–60 days of culture. The massive initiation of embryos is crucial for obtaining high gynogenic embryo numbers and increases the probability of haploid plant production (Ibrahim et al. 2016). The time of gynogenic embryo initiation was significantly variable between different auxin treatments and ecotypes. Genotype proved to be one of the most important factors affecting in vitro gynogenesis in alliums (Ibrahim et al. 2016; Sulistyaningsih et al. 2006).

Table 1. Effect of auxin type and concentration and ecotype on gynogenic embryo induction and callus formation in unpollinated flower cultures of *Allium hirtifolium* on the medium containing 2 mg·dm^{-3} benzylaminopurine (BAP)

Ecotypes	Auxin (mg·dm^{-3})	Days to gynogenesis induction	Gynogenesis induction (%)	Number of gynogenic embryos per flower	Callus induction (%)
Arak	2,4-D (1)	57.64±0.33 ef *	5.91±0.02 i	0.79±0.21 j	19.33±0.96 e
	2,4-D (2)	58.94±0.57 bc	7.67±0.1 gh	1.14±0.11 i	27.22±0.85 c
	NAA (1)	56.30±0.38 g	8.55±0.05 efg	1.20±0.23 h	9.09±0.62 p
	NAA (2)	58.16± 0.56 cde	9.42±0.08 cde	1.46± 0.21 c	11.22±1.01 k
Lorestan	2,4-D (1)	58.63±0.32 bcd	7.74±0.11 gh	0.81± 0.31 j	17.22±0.52 f
	2,4-D (2)	57.86±0.12 def	8.59± 0.12 efg	1.36±0.22 e	28.24± 0.69 b
	NAA (1)	57.25± 0.88 ef	9.67±0.09 cd	1.42±0.09 d	9.23±0.85 o
	NAA (2)	57.23± 0.63 ef	12.31±0.05 a	1.64±0.11 a	10.15±0.42 m
Sanandaj	2,4-D (1)	59.33±0.33 b	7.14±0.03 h	0.71±0.44 k	16.33±0.32 g
	2,4-D (2)	58.66±0.54 bcd	7.88±0.21 gh	1.15±0.31 i	29.16± 0.74 a
	NAA (1)	56.06±0.62 g	8.37±0.11 f g	1.22±0.11 h	10.52±0.84 l
	NAA (2)	57.19±0.23 f	10.25±0.21 bc	1.47±0.20 c	12.24±0.65 j
Yasoj	2,4-D (1)	59.42±0.11 b	7.27±0.03 h	0.73±0.16 k	15.45±1.12 h
	2,4-D (2)	60.69± 0.58 a	7.84±0.05 gh	1.26±0.10 g	26.34±0.65 d
	NAA (1)	57.42±0.72 ef	9.06±0.06 def	1.31±0.21 f	9.64±0.53 n
	NAA (2)	58.17±0.52 cde	10.95±0.1 b	1.56±0.31 b	13.40±0.84 i

* Values followed by the same letter within a column indicate they are not significantly different ($p < 0.05$) ±SD.

Yasoj ecotype showed later signs of embryo induction than other ecotypes. The shortest time required for gynogenic induction were observed in culture media containing 1 mg·dm^{-3} NAA and the longest in cultures in medium with 2 mg·dm^{-3} 2,4-D (Table 1). The emerged embryos were developed to whole plants after 2–3 weeks. The percentage of gynogenesis induction in this study ranged from 5.9 to 12.3% depending on the treatment. The highest percent of gynogenic induction in cultured flowers was obtained in Lorestan ecotype in the medium containing 2 mg·dm^{-3} NAA. The percent of gynogenic embryo induction and gynogenic plantlet numbers in media containing NAA was higher than in culture media with 2,4-D. All genotypes reacted in the same way on growth regulators. Percent of gynogenesis induction and number of gynogenic embryos per flower was higher on media containing NAA than on 2,4-D and the highest on medium containing 2 mg·dm^{-3} NAA. The most productive was Lorestan ecotype with 12.3% of induction and 1.6 embryo per flower. The same proportion of BAP and NAA was most effective in gynogenesis of *Allium cepa* (Campion et al. 1992). Differences in the requirement for growth regulators might be due to the differences in the explant type and genotype (Juðkevièienë et al. 2005). In most cases, one gynogenic embryo is obtained from one responsive flower (Alan et al. 2004); however, in our experiment, more than one embryo was obtained from some ovaries, similarly as in the report of Shalaby (2007). Previous reports in alliums have shown a strong variation in gynogenic embryo induction between genotypes (Hassandokht et al. 2000). Michalik et al. (2000) reported that the onion cultivars differ with regard to their requirement for media composition, and gynogenetic embryo induction depends on the genotype.

In this study, the plantlets derived by somatic organogenesis were obtained by both directly from explant tissue and indirectly from callus. The plantlets formed outside the ovaries were observed in all cultured flowers and their number was not significantly different between ecotypes. The lowest (6.0) number of directly regenerated plantlets from outside the ovaries was recorded on media containing 2 mg·dm^{-3} BAP + 2 mg·dm^{-3} 2,4-D, respectively (Fig. 1).

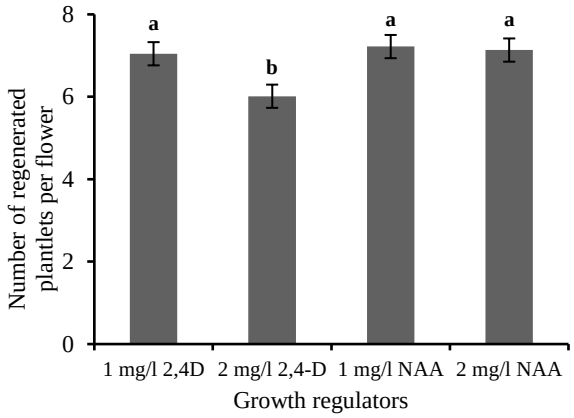

Fig. 1. Effect of auxin on number of directly regenerated plantlets outside the ovaries in unpollinated flower cultures of *A. hirtifolium* on medium containing 2 mg·dm^{-3} BAP. Values followed by the same letter indicate they are not significantly different ($p < 0.05$) ± SD

The three other media gave a bit more than seven regenerated plantlets per flower. Luthar and Bohanec (1999) and Sulistyaningsih et al. (2006) reported that direct somatic organogenesis could be induced in unpollinated flowers or ovaries of common onion and shallot. In our study on Persian shallot, there was no relationship between the capacities for gynogenesis and somatic organogenesis.

The highest callus formation in all ecotypes was observed on the medium containing 2 mg·dm^{-3} 2,4-D. Sanandaj ecotype showed the highest callus induction (29.2%) and the lowest percent (9.1%) was observed in Arak ecotype on the medium containing 1 mg·dm^{-3} NAA (Table 1). In this study, similarly as in the reports of Bohanec and Jakše (1999), the formation of basal callus was not correlated with lower embryo yield, since the ecotypes with highest and lowest callus induction showed the same gynogenic embryo response.

The obtained calli were transferred to shoot induction media containing 1.5 mg·dm^{-3} BAP and 0.5 mg·dm^{-3} NAA. The plantlets regenerated from 51.6, 53.3, 57.9, and 64.8% of calli, from Yasoj, Lorestan, Sanandaj, and Arak ecotypes, respectively (data not shown). Figure 2 shows the process of plantlets regeneration from not generative parts of flowers of *A. hirtifolium* of Lorestan ecotype.

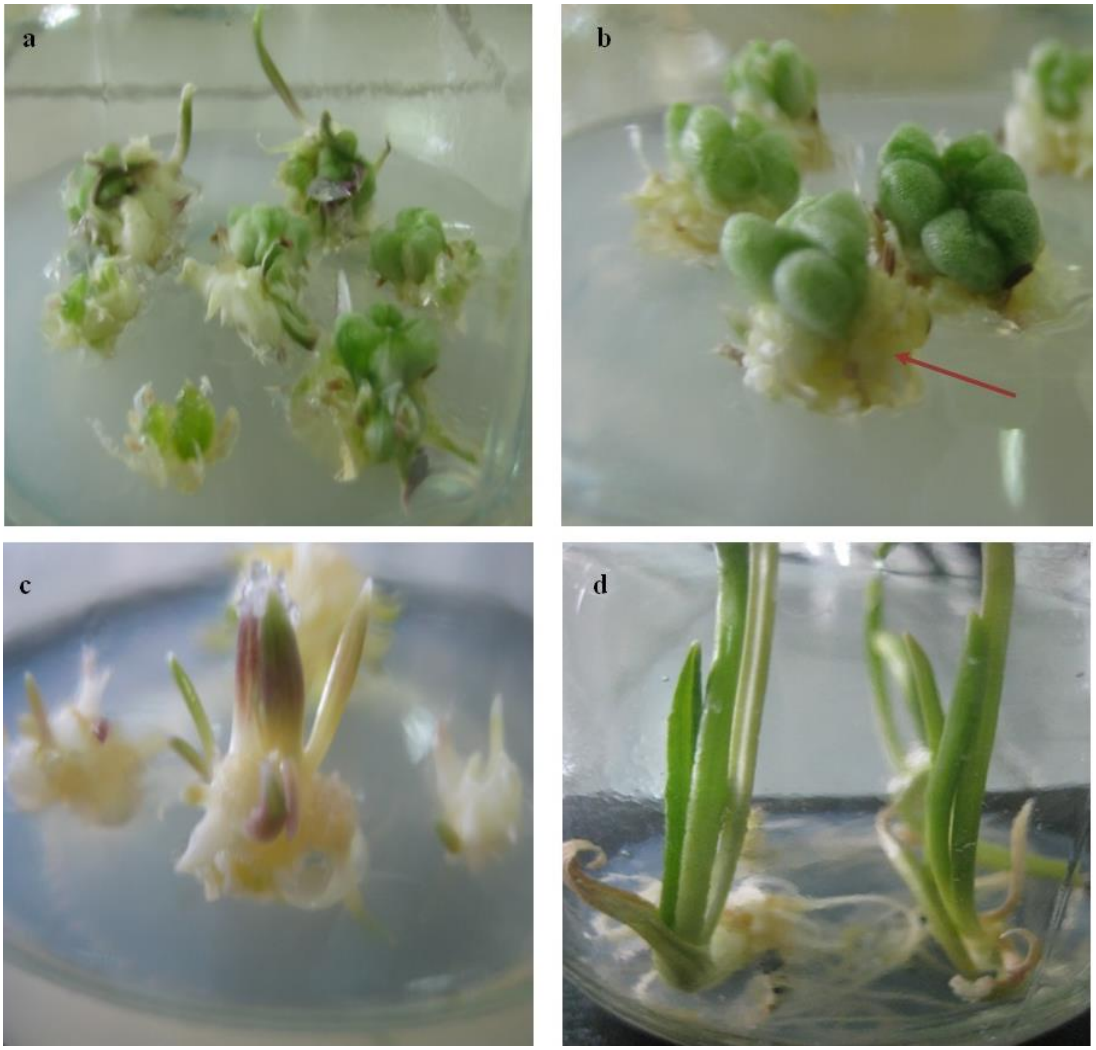

Fig. 2. Plantlets' development from cultures of unfertilized flowers of Lorestan ecotype of *A. hirtifolium*: (a) the regenerated plantlets in medium containing 2 mg·dm^{-3} BAP + 2 mg·dm^{-3} NAA; (b) callus formation in flower cultures; (c) callus-derived plantlets from somatic organogenesis in MS media supplemented with 1.5 mg·dm^{-3} BAP and 0.5 mg·dm^{-3} NAA, (d) root formation in regenerated plantlets on hormone-free MS medium.

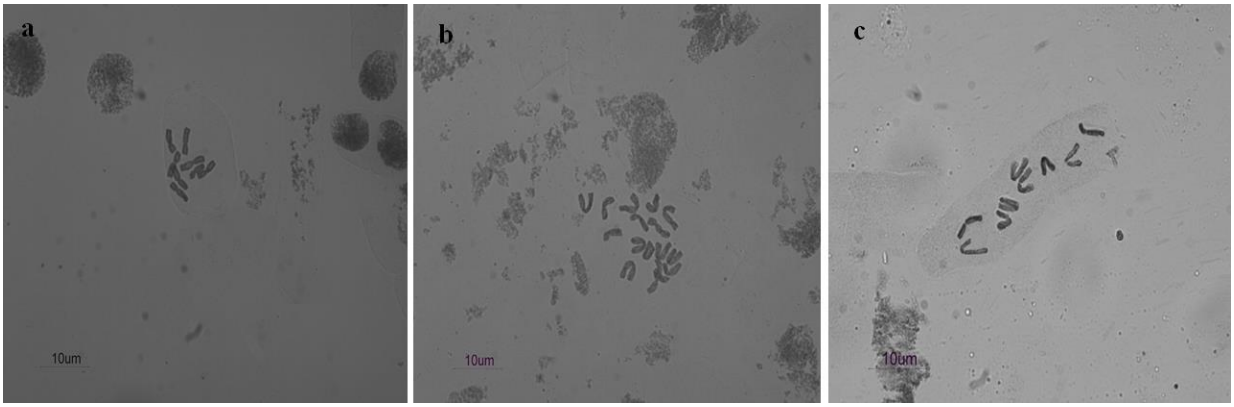

Fig. 3. Chromosome numbers in plantlets from gynogenesis of *A. hirtifolium* in Lorestan ecotype. (a): haploid (2n = x = 8), (b): diploid (2n = 2x = 16), (c): disomic plantlet (2n = 8 + 1)

Table 2. Chromosome numbers of gynogenic, directly regenerated, and callus-derived plantlets of *A. hirtifolium*

Ecotypes	Frequency of plantlets with different chromosome numbers (%)		
	diploid (2n = 16)	haploid (2n = 8)	mixoploid (2n = 8, 16)
Gynogenic plantlets			
Arak	6	75	19
Lorestan	3	77	18
Sanandaj	2	76	22
Yasoj	8	70	22
Directly regenerated plantlets			
Arak	100	0	0
Lorestan	80	0	20
Sanandaj	78	0	22
Yasoj	81	0	19
Callus-derived plantlets			
Arak	79	11	10
Lorestan	70	13	17
Sanandaj	71	9	20
Yasoj	77	10	13

As shown in Table 2, 70–77% of plantlets obtained from gynogenetic embryos were haploid with 8 chromosomes (Fig. 3a); 2–8% were diploid with 16 chromosomes (Fig. 3b) and the rest were mixoploid (Fig. 3c). The mixoploid plants showed chromosome numbers of haploid or diploid level in the same root or in different roots. Percent of haploid plants did not depend on ecotype (70–77%). The mean percentages of embryo induction, which developed into complete haploid plantlets in onion (Alan et al. 2004), long-day onion (Bohanec & Jakše 1999), spring onion (Ibrahim et al. 2016), leek (Kaska et al. 2014), and shallot (Sulistyaningsih et al. 2006) were 82, 90.5, 0.5, 55.6, and 20, respectively. Frequency of haploid induction may be influenced by genetic, environmental, and physiological factors (Alan et al. 2004).

Several authors reported obtaining of diploid plants of allium from cultures of unpollinated flowers (Alan et al. 2004; Bohanec & Jakše 1999; Ibrahim et al. 2016; Kaska et al. 2014; Sulistyaningsih et al. 2006). The induction of haploid plants originates from gametophytic cells that have undergone meiosis, and the presence of diploid plantlets can be the result of spontaneous diploidization during embryo formation in the ovule or later. So, it is possible that diploid plants obtained from gynogenesis are indeed doubled haploids. Marker analysis is needed to support this possibility, and if it is confirmed, the obtained doubled haploid plants can serve as pure lines in breeding programs (Alan et al. 2004; Campion et al. 1992; Sulistyaningsih et al. 2006). It is considerable that we found 2% of disomic plantlets (2n = x + 1 = 9) among regenerated gynogenic plants of Lorestan ecotypes that would be an interesting material for genetic and cytogenetic studies in this less known *Allium* species (Fig. 3c). Sulistyaningsih et al. (2006) reported a trisomic cell (2n = 2x + 1 = 17) from a callus-derived plant in shallot (*A. cepa* L. Aggregatum group).

The directly regenerated plants and callus-derived plants showed high share of diploid cells (Table 2) but within plantlets directly regenerated no haploid cells were found, whereas within those regenerated from callus the haploid constituted about 10%. Gynogenic plantlets were mostly haploid. Mixoploidy was rather randomly distributed in all ways of regeneration. The different chromosome numbers in regenerated plantlets from somatic organogenesis were also reported by Sulistyaningsih et al. (2006). They reported that the concentrations of plant growth regulators in the cultured medium

and prolonged in vitro culture were the possible reason of somaclonal variation revealing in the number of chromosomes.

CONCLUSION

Our success in haploid plantlets production using unfertilized flower buds showed that in vitro gynogenesis is an effective alternative way to speed up the conventional inbreeding process of Persian shallot (*A. hirtifolium*). Further studies need to determine the origin of diploid plants whether they are doubled haploids or originate from somatic tissues, or from rarely occurring unreduced eggs. Further studies are also needed to produce double haploid plants by duplication of the number of chromosomes in haploid plants that enabled faster production of homozygous lines for breeding of new cultivars of *A. hirtifolium*.

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