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Research Article
Haploid Induction in Spring Onion (*Allium fistulosum* L.) via Gynogenesis

[1]Ahmed Mahmood Ibrahim, [1]Fatimah Binti Kayat, [1]Dwi Susanto, [2]Pedram Kashiani and [1]Mohammed Arifullah

[1]Faculty of Agro Based Industry, University Malaysia Kelantan, Jeli Campus, Locked Bag 100, 17600, Jeli, Kelantan, Malaysia
[2]Department of Crop Science, Faculty of Agriculture, University Putra Malaysia, UPM Serdang, 43400, Selangor, Malaysia

Abstract

Regeneration into haploid plantlets had been obtained in spring onion using flower and ovary culture. Flowers and ovaries were cultured into media using two protocols (A and B) and the ability to produce callus or somatic embryogenesis were invistigated. Flowers around 3.0-5.0 mm long were collected, whole flower or ovary which were excised from flowers using dissecting microscope were cultured into BDS medium supplemented 2.0 mg L^{-1} 2, 4-D and 2.0 mg L^{-1} BAP fortified with 100 g L^{-1} sucrose, 200 mg L^{-1} proline, 500 mg L^{-1} myo-inositol (protocol A) or into BDS media supplemented 2.0 mg L^{-1} 2,4-0 and 2.0 mg L^{-1} BAP for 14 days, then sub-cultured by regeneration medium (BDS) supplemented with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} 2iP (protocol B). Embryos were emerged from ovary wall after around 4-5 months, high frequency of embryogenesis induction was produced from ovaries that were using protocol A. While the less percentage observed from flower culture using protocol B.

Key words: Ovule, ovary, embryogenesis, BDS media, shoot regeneration

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Corresponding Author: Fatimah Binti Kayat, Faculty of Agro Based Industry, University Malaysia Kelantan, Jeli Campus, Locked Bag 100, 17600, Jeli, Kelantan, Malaysia

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Spring onion (*Allium fistulosum* L.) is a biennial monocotyledonous plant is one of the most important vegetable crops around the world in terms of crop value. Spring onion is grown widely in Cameron Highlands particularly in the Tringkap and Kea Farm areas (Ramasamy, 1991). The demand for specific varieties accommodate to local environment is too high since onion is a photoperiodically sensible plant and forms bulbs only after special environmental conditions among genotypes. According to the inbreeding method, two types of onion varieties are cultivated: hybrid varieties and open pollinated (Jakse and Bohanec, 2003). Approximately 170 countries grow onions for their own domestic use and many are also involved in international trade. Onion ranking coming second after tomato in the list of vegetables cultured worldwide, It is estimated that over 90 million tons on 4.7 million ha (FAO., 2013). The production of F_1 hybrids is considered one of the main goals in onion breeding program. The main restriction of this field is the length of the time needed to produce inbred. The most time-consumption and work-intensive aspect of improvment these hybrids is the conventional breeding process that requires manual self-pollination essential to generate pure lines of homozygous parent. This way requires eight or more off-springs of inbreeding to establish sufficient regular lines that can be applied in hybrid production. In many varieties *in vitro* methods have provided to speed up the development of homozygous lines, as an alternative to the slower conventional inbreeding process. Haploid plants may be accomplished by using anther, unfertilized ovule, ovaries or entire flowers buds culture. The number of haploid chromosome means that meiotic recombination's and recessive gene effects are manifested at plant level. And spontaneous or induced chromosome doubling consent the regeneration of doubled haploids (DHs) homozygous materials, with restored fertility that can be used in different breeding strategies because all the alleles of DHs lines are fixed and aid efficient selection for quantitative traits in breeding. Induction of superior onion hybrids rely on the advancement of high-quality, fertile inbred lines to use as parents. These lines should be sufficiently inbred, because commercially suitable hybrids should be uniform for horticultural characterizes such as bulbs shape, bulbs size and time to maturity. By rising homozygosity of the inbred parent lines this uniformity can be achieved through cycles of self-pollination. However, such as many cross-pollinated crops, onions suffer from hard inbreeding depression when self-pollinated for several generations (Bohanec, 2002). Female gametophyte (gynogenesis) is an alternative method in onion for haploid creation. Several authors has been reported successfully by (Bohanec *et al*., 1995; Geoffriau *et al*., 1997; Martinez *et al*., 2000; Michalik *et al*., 2000; Fayos *et al*., 2015). Geoffriau *et al*. (1997) reported that the main limitation of gynogenesis in onion is the low percentage of embryogenesis formation, chromosome doubling and plant survival from most of the materials.

The first research on onion gynogenesis were applied with ovule and ovary culture, most of cases, protocol from two step was applied, a preculture of the flower buds prior ovule or ovary excised. In these studies MS (Murashige and Skoog, 1962), B5 media (Gamborg *et al*., 1968) and BDS (Dunstan and Short, 1977) were used (Muren, 1989; Keller, 1990; Campion and Alloni, 1990; Campion *et al*., 1992). later, new protocols were improvment dependce on the first protocol on those used for ovule and ovary culture with some modifications on culture medium, including PGR (Bohanec *et al*., 1995; Martinez *et al*., 2000; Michalik *et al*., 2000), afterward on a simplified by one step protocol, containing of culturing the completed flower bud in an induction media until the embryo induction (Bohanec and Jakse, 1999; Jakse and Bohanec, 2003).

The objective of this study was to evaluate gynogenesis ability of spring onion gynotype using flower bud and ovary culture and to optimize the frequency of embrayo induction and acclimated haploid plants. two protocols, (A) described by Jakse and Bohanes (2003) and (B) described by Michalik *et al*. (2000) were examined in this study.

MATERIALS AND METHODS

This study was carried out in the tissue culture laboratory of Faculty of Agro Based Industry, University Malaysia Kelantan.

Plant material and sterilization: Whole umbel of spring onion (*Allium fistulosum* L.) was taken from donor plants when the flowers were at 3-4 days before anthesis (Fig. 1), individual flower 3-5 mm with pedicel were removed and sterilized with 70% alcohol for 45 sec then surface-sterilized with 5% sodium hypochlorite (NaClO) with a few drops of tween 20 for 15 min and rinsed four times with sterile distilled water.

Culture media: Two protocols were used in this study, first protocol (A) described by Jakse and Bohanes (2003) was followed in this study, whole flower buds or ovaries after excised from flower were cultured in Petri dish 100×15 mm

Fig. 1: Plant material of spring onion, umbel 3-4 days before anthesis

contained BDS medium (Dunstan and Short, 1977) supplemented 2.0 mg L^{-1}, 2, 4-d and 2.0 mg L^{-1} BAP fortified with 100 g L^{-1} sucrose, 200 mg L^{-1} proline, 500 mg L^{-1} myo-inositol, 10 mg L^{-1} adenine sulfate and vitamins, pH was adjusted to 5.8 prior to addition 6 g L^{-1} agar, then autoclaved for 15 min at 121°C. Ten flowers or ovaries were inoculated in each Petri dish and placed in growth room with light illumination (16/8 h) under 25°C until embryo production. The percentage of gynogenic embryos, entire plants and haploid plantlets were scored and percentage of acclimated. Second protocol (B) described by Michalik *et al.* (2000), flowers or ovaries were inoculated into BDS media supplemented 2.0 mg L^{-1} 2,4-0 and 2.0 mg L^{-1} BAP for 14 days, then sub-cultured by regeneration medium (BDS) supplemented with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} 2iP. Embryo was broken through the ovary wall was sub cultured into to medium having MS media without growth regulators and supplemented with 30 g L^{-1} sucrose until plant development.

***In vitro* rooting and acclimatization:** Embryo formation is awaited about four to six months in culture. Regenerated plants were recorded around 130 days. At that time of embryo emergence mostly of ovaries will change color from green to yellow. New embryos were transferred to MS media half strength, supplemented 1.0 mg L^{-1} IBA+1.0 mg L^{-1} KIN with 0.5% charcoal for root induction and shoot development for 3-4 weeks, then were transferred to tubes having tap water and covered by piece of cotton and put in growth room for 1-2 weeks to help for autotrophic. Next step new plantlets were transferred to 7 cm plastic pots containing peat moss and covered with plastic cap having small hole in top for gaseous exchange for one weeks and we started remove the cap for one hour per days and increase uncovered time daily two, three and four hours for two weeks, finally the cap was removed.

Flow cytometry: Flow cytometer BD Accuri™ C6 was used for ploidy test and the following procedure were used to isolate the nuclei from haploid and diploid onion leaf (a) 150 mg of onion leaves were weighted, (b) Covered with 1 mL ice-cold tris $Mgcl_2$ buffer (200 mM tris, 4 mM MgCl 2.6 H_2O, 0.5% (v/v) triton X-100, pH 7.5), (c) Chopped with scalpel edge blade, worked quickly, (d) Added 1 mL PI/RNase staining and mix promptly and kept it in cool and dark place for 2 h before run and (e) Filtered the suspension through a 40 µm mesh nylon filter in ice-cool cytometer sample tube and run the sample. Leaves from young seedlings were used as control (Galbraith *et al.*, 1983).

Data analysis: All experiments were designed according to Completely Randomized Design (CRD). The data was analyzed using one-way analysis of variance (ANOVA) using the General Linear Model (GLM) and *post hoc* multiple comparisons for observed mean, significant differentiations were compared by Duncan Multiple Range Test (DMRT).

The following Eq. 1-3 were used to calculated percentage of callus, shoot and root induction:

$$\text{Callus induction} = \frac{\text{No. of callus induction}}{\text{No. of explants}} \times 100 \quad (1)$$

$$\text{Shoot induction} = \frac{\text{No. of shoot induction}}{\text{No. of calli}} \times 100 \quad (2)$$

$$\text{Root induction} = \frac{\text{No. of root induction}}{\text{No. of calli}} \times 100 \quad (3)$$

RESULTS

Haploid production: The flowers, ovaries, anthers and ovules excised from the chosen developmental stage were subjected to two different protocols (A and B) before being cultured onto BDS media. Flowers began to bloom after 4-5 days cultured in BDS medium and swelled up to two times of their original size. Since there was no anther opening being observed, it was assumed that self pollination do not occur. Callus or shoot formation appeared after four to six months of inoculation. Calli were recorded around 90 days after flower

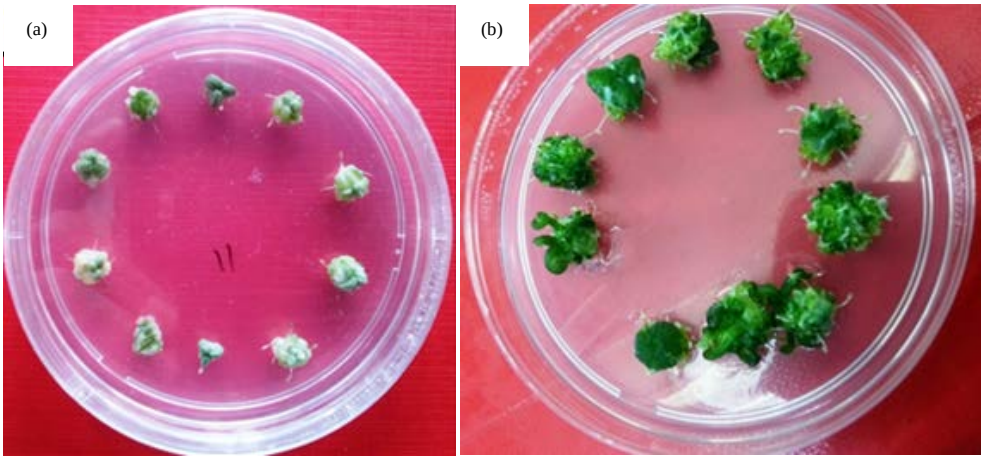

Fig. 2(a-b): (a) Calli produce from the ovary cultures after 90 days in BDS media and (b) Shoots regeneration observed from the ovary cultures after 60 days in BDS media

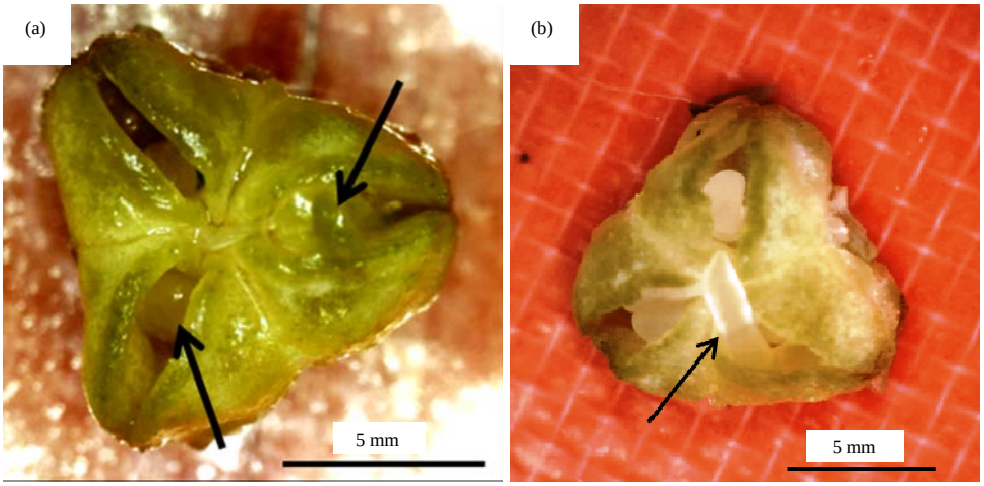

Fig. 3(a-b): (a) Callus induction from ovule after 90 days and (b) Shoot induction after 60 days of callus induction

Table 1: Effect of two types of media on callus induction of flower, ovary and anther culture from spring onion

			Calli induction		Shoot induction		Acclimated plants	
Culture media	Expnats	No. of explants	No. of calli	Calli/flower (%)	Embryos	Embryos/flowers (%)	Plants	Plants/embryo (%)
Protocol A	Flower	650	5	0.77[b]	3	0.51[b]	1	33.3
	Ovary	300	7	2.33[a]	4	1.52[a]	2	50.0
Protocol B	Flower	550	5	0.91[b]	3	0.69[b]	0	0.0
	Ovary	250	6	2.40[a]	4	1.33[a]	2	50.0

Values followed by the same letters within a column are not significantly different (≤0.05), Duncan's Multiple Range Test (DMRT)

and ovary inoculation and shoot induction was observed after 60 days of callus induction in BDS media (Fig. 2). The cultures were green in color during the first three months and gradually turned to yellowish as the culture progressed (Fig. 3). Calli from the ovule were easily recognized with burst of ovary after 4-5 months of inoculation.

Results obtained showed that there was a significant difference in the percentage of callus produced from the two protocols. Higher percentage of callus induction had been recorded for ovary culture by using protocol B with 2.4% compared to protocol A with 2.33%. The flower culture also showed higher percentage of callus induction in protocol B compared to protocol A with 0.91 and 0.77%, respectively (Table 1). However, no callus being produced for the anther cultures in both protocol A and B. The shoot regeneration had been observed from the callus produced (Fig. 4). A significant

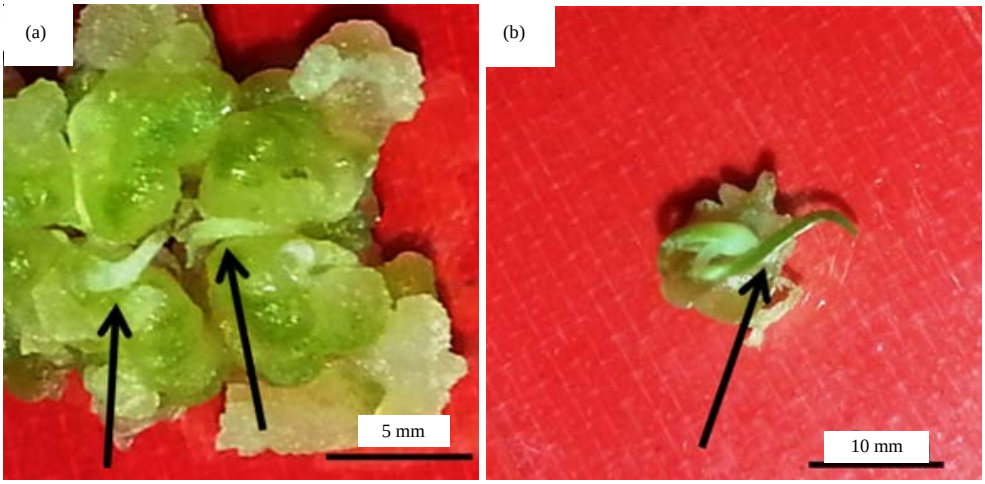

Fig. 4(a-b): Shoots regeneration observed from callus of the ovule culture (b) Shoot development observed after 150 days of culture

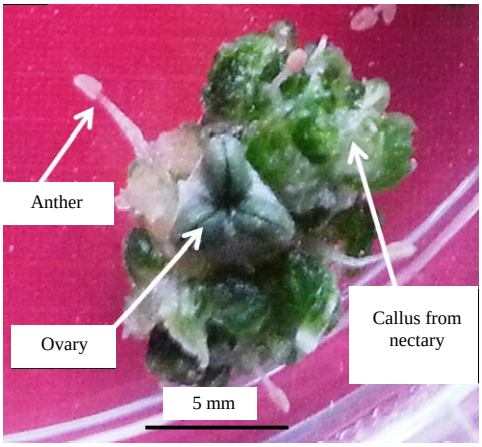

Fig. 5: Callus and shoot regeneration from the septal nectaries region of the flower culture (discard)

response towards shoot regeneration had also been observed with 1.52% for protocol A and 1.33% for protocol B while, the percentage of shoot produced from the flower culture was higher in protocol B compared to protocol A with 0.51% and 0.69%, respectively (Table 1). No shoot being produced from the anther cultures for both protocols. Beside calli production and shoot regeneration from the flower culture, direct shoot regeneration had also been observed from the septal nectaries region of the flower (Fig. 5).

***In vitro* rooting and acclimatization:** Root and shoot development had been obtained by using half strength MS media supplemented with 1.0 mg L^{-1} IBA+1.0 mg L^{-1} KIN and added with 0.5% activated charcoal. Rooted plantlets produced form both protocols were acclimatized in tubes

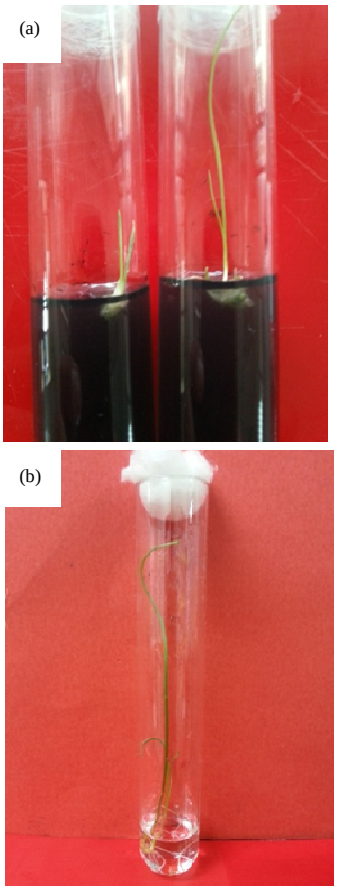

Fig. 6(a-b): (a) *In vitro* rooting and (b) Acclimatization of plantlets produce from both protocols

added with tap water to decrease plant hyper hydricity (Fig. 6). Then, the acclimatized plantlets were let to grow for ploidy determination (Fig. 7).

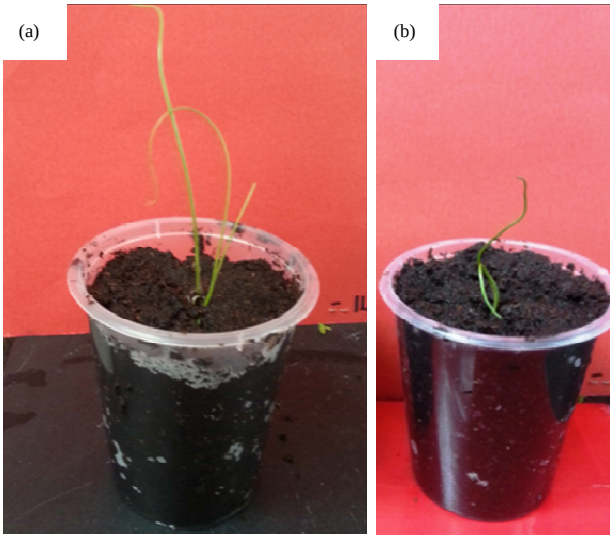

Fig. 7(a-b): Acclimatized haploid and diploid plants, (a) Diploid and (b) Haploid

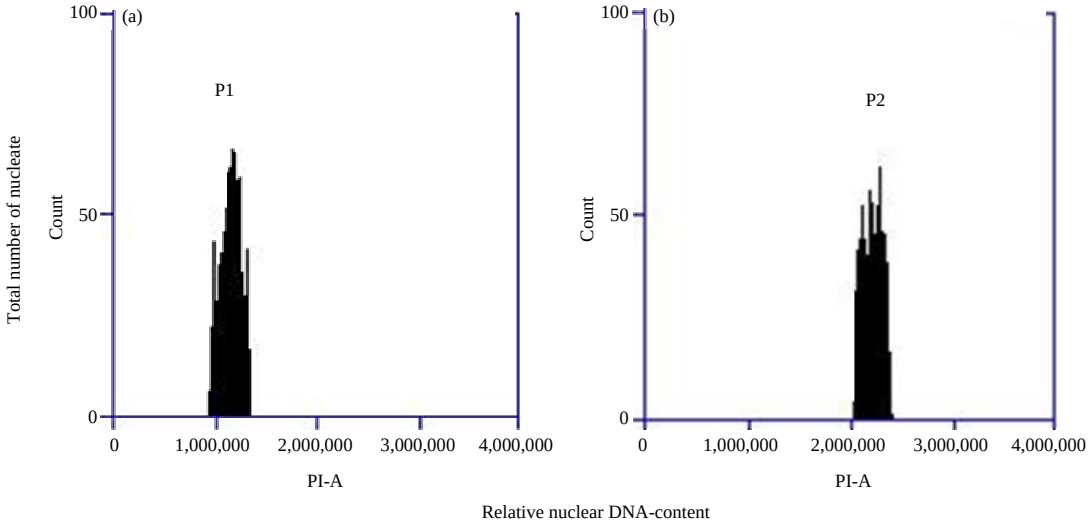

Fig. 8(a-b): Flow cytometry profiles showing the nuclear DNA content of the spring onion plantlets, (a) Haploid (n) and (b) Control (2nd) diploid

Ploidy test: Leaves of spring onion were collected and their ploidy was examined by flow cytometry. The flow cytometry profiles of the plantlets generated from ovule culture showed a single peak at around 1.000 PI-A (P1) while, the plantlets generated from diploid (control) showed a single peak at around 2.000 PI-A (P2) (Fig. 8). Therefore, it is confirmed that plantlets generated from the ovule culture contained half of DNA content compared to plantlets generated from diploid. Based on the amount of DNA content, it is appeared that plantlets generated from the ovule culture were haploid, by comparing to the control diploid control plantlets.

DISCUSSION

Gynogenesis capacity from spring onion has been evaluated and two protocols have been assayed in order to produce haploid plant. Genetic factors, including varieties, geographic origin, plant genotype and genetic structure are believed to be very important for the achievement of gynogenesis formation (Campion and Alloni, 1990; Geoffriau *et al*., 1997; Bohanec and Jakse, 1999; Michalik *et al*., 2000; Chen *et al*., 2011). Bohanec and Jakse (1999) found indicating of gynogenesis is strongly affected by the

genotype. In the present study, the addition of proline with BAP+2, 4-D to the medium induced the onset of embryogenesis and raise the amount of gynogenic embryos gained. The embryo initiation phase is crucial to develop the yield of regenerated plantlets as the higher gynogenic embryo number and the great probability of getting haploid plants. The mean percentage of embryos induction, which developed into entire haploid plantlets was 0.46%. This confirm the results of Bohanec *et al.* (1995), Phillips and Luteyn (1983) and Geoffriau *et al.* (1997), who demonstrate that the haploid plants production in onion was quite affected by the population. Genetic factors, including variety, geographic origin, genotype of donor plant and genetic structure are thinking to be the high importance for the success of gynogenesis production (Campion and Alloni, 1990; Geoffriau *et al.*, 1997; Bohanec and Jakse, 1999; Michalik *et al.*, 2000; Chen *et al.*, 2011). In Spanish germplasm, variety and plant genotype as well has a strong effect on gynogenesis formation, two Valencia-type varietes Rita and Recas exhibted the highest embryogenesis frequency (0.87-2.09%) then, pungent landrace BGHZ1354 (0.40-1.28%) and lastly the sweet cultivar Fuentes de Ebro (0.27-0.75%) (Fayos *et al.*, 2015).

CONCLUSION

Our successful haploid induction plant from spring onion using whole flower buds and ovaries allow the use this method in programs of onion breeding, further studies need to produce double habloid plants by dublicate number of chromosome in haploid plants to fast homozygous ilnes production for new varieties production.

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