Madras Agric. J., 2019; doi:10.29321/MAJ 2019.000257

RESEARCH ARTICLE

Haploid Induction in Multiplier Onion (*Allium cepa* var. *aggregatum*) through *in vitro* Gynogenesis

Adhiyamaan, S[1], Arumugam, T[2], Gnanam, R[3] and Shoba Thingalmaniyan, K[1]
[1]Department of Vegetable crops, HC&RI, Tamil Nadu Agricultural University, Coimbatore- 641 003
[2]Horticultural College and Research Institute, TNAU, Periyakulam- 625 604
[3]Department of Plant Biotechnology, AC&RI, Tamil Nadu Agricultural University, Coimbatore- 641 003

Received : 20th May, 2019
Revised : 6th June, 2019
Accepted : 6th June, 2019

ABSTRACT

The haploid induction study is an effective method for inbred and hybrid development quickly. The optimization of media composition for specific indigenous genotypes was a critical factor. Two media namely, Gamborg's medium (B5) and Murashige and Skoog's medium (MS) with six different combinations of plant growth regulators were used to evaluate the gynogenic potential of six different genotypes of multiplier onion. Days for embryo induction was earlier in Acc. 1 (46-54), whereas Acc. 4 was the best responding genotype with a higher percent of embryo induction (0.76) followed by Acc. 15 (0.48). The embryo induction rate and survival rate of 0.21% and 54.54% respectively were recorded in B5 medium whereas in MS medium the induction and survival rate was 0.34 % and 34.86 % respectively. Embryo induction ranged from 0 to 1.41% in this study. Five haploids and one mixoploid in B5 medium, five haploids in MS medium were obtained. Ploidy status of the plants was confirmed by flow cytometry, cell cytology, and stomatal dimension analysis.

Keywords: *Multiplier onion (Allium cepa var. aggregatum), In vitro gynogenesis, Embryo, Haploid, Flow cytometry, Chromosome counting, Stomatal dimension difference.*

ABBREVIATIONS

ACC. Accession
HM Haploid Medium
2,4-D 2,4-Dichlorophenoxyacetic acid
NAA a-Naphthaleneacetic acid
BA 6-Benzylaminopurine
2-iP 2-Isopentenyladenine

INTRODUCTION

Onion (*Allium cepa*) is an important vegetable crop of Alliaceae family which is having a higher foreign exchange value of 30.9 billion rupees among fresh vegetables cultivated in India. Onion also has medicinal properties include antifungal, antioxidant activity and it prevents atherosclerosis and coronary heart disease. India stands second position (17% total production) after China. Onion hybrid development through the conventional breeding process is extended about 10-12 years, and several generations of selfing were difficult due to high inbreeding depression and biennial nature. Complete homozygosity is imperative for any heterosis breeding while onion genotypes are not fully homozygous due to high cross-pollination. India endowed less productivity was primarily due to the cultivation of short day Indian onion and less realization of hybrid vigour (Manjunathagowda *et al*. 2015). Hybrids perform better in all aspects than open-pollinated varieties but only later dominated among farmers cultivation. Further, the complex nature of inheritance, less QTL known for biotic and abiotic factors made onion research difficult. The inbred development through *in vitro* gynogenesis technique address this issue within two years, which involves the development of doubled haploid plants by chromosome doubling treatment. *In vitro* anther culture technique has most used for haploid induction in many angiosperms (Germana *et al*. 2001) but, it was not successful in onion (Roy . 1989; Keller *et al*. 1990).

Most important gene concerning abiotic stress was upregulated in double haploid of *A. cepa* var *aggregatum* and F_1 genotypes than doubled haploid common onion in the transcriptome analysis. Multiplier onion research was not focused much in India though demand for key genes and market exists (Abdelrahmen *et al*. 2015). In India, the export of multiplier onion recorded 50000 MT during 2016-17 (Anonymous. 2018). Sufficient doubled haploid population is needed for breeding to introgression of an important gene into cultivated form. The aim

*Corresponding author's e-mail: tarumugam64@gmail.com

of this research was to identify multiplier onion genotypes that have greater gynogenic potential and selective media for unfertilized flower culture. This research forms the base for the development of hybrids within a short time.

MATERIAL AND METHODS

Plant material

Six short day multiplier onion (*Allium cepa* var. *aggregatum*) were selected based on 100 per cent bolting behavior (table 1), all of which were local accession collected in Tamil Nadu, India. Bulbs were grown under open field condition during *Kharif* season-2018 at ICAR-Directorate of Onion and Garlic Research, Pune, Maharashtra.

Flower culture

The unopened flower buds before three days to anthesis from 30 % flower opened umbel were used as explant (fig. 1a) and the number of explants was given in table 4. Surface sterilization was done by immersion of flower bud in 70% ethanol for 5 minutes followed by rinsing three times with water. Then, the explants were treated with 0.1% mercuric chloride for 5 minutes along with tween 20 wash, finally rinsed with water for five times under sterile condition.

B5 and MS basal medium supplemented with the different combination of plant growth regulators and polyamines were used (table 2). The pH of all the media was adjusted to 5.8 and then autoclaved. The flower culture (fig. 1b) was maintained at 25 ˚C with 60 percent relative humidity and 16 hours light, 8 hours dark cycle period with the light intensity of about 4000 lux. After shoot emergence, the gynogenic embryos were transferred to a test tube containing half MS medium (fig. 1i). The protocol for haploid induction in multiplier onion has been given in step by step in figure 1.

Observations

The parameters on (1) number of flower bud inoculated, (2) number of days to opening of flower buds, (3) percentage of flower bud opening, (4) percentage of gynogenic plantlets, (5) percentage of plantlets survived, (6) number of haploid plants, (7) days to embryo response, (8) percentage of callus formation, (9) percentage of somatic organogenesis, (10) percentage of non-responsive bud including dead buds and abnormally enlarged buds (plumpy) were recorded.

Ploidy level determination

The ploidy status of regenerated plants was determined by flow cytometry, chromosome counting and stomatal dimension differences.

Flow cytometry

Leaves from the putative haploid plants were chopped in ice-cold Galbraith buffer and stained with propidium iodide (PI) for flow cytometry analysis in BD Accuri ™ C5 instrument.

Chromosome counting

Meristematic roots were treated in 0.05 % colchicine for 3 hours and fixed in a mixture of ethanol and acetic acid (3:1 v/v). After hydrolysis in 1N HCl at 65 ° C for 7 minutes, they were stained with 1 % acetocarmine solution. Chromosome number analysis was done using a compound microscope at 40 X magnification.

Stomatal dimension difference

A thin layer of epidermal leaf cells was peeled off, treated with acetocarmine solution and the stomatal dimension difference between haploid and diploid plants were compared through LEICA DM 2500 microscope software.

RESULTS AND DISCUSSION

Effect of explant

The flower bud size taken for an experiment between 2.5-4.0 mm recorded as a better response than larger (>4.5 mm) and smaller (<2 mm) buds. The flower buds stored in 4°C had persistent response and equivalent to the fresh flower buds.

Table 1. Characters associated with genotypes

S.No.	Genotypes	Total yield (t/ha)	Thrips damage (%)	Purple blotch (PDI)	Stemphyllium Blight (PDI)	Bulb shape	TSS (%)	Bulb colour
1	Acc. 4	17.7	28.4	19.0	2.9	R/O	15.1	Pink
2	Acc. 9	20.8	17.4	11.9	2.1	R/O	17.6	Pink
3	Acc. 15	22.4	8.8	9.7	4.7	R/O	18.0	Pink
4	Acc. 1	18.4	25.4	7.5	4.5	R/O	15.2	White
5	Acc. 2	18.3	28.8	20.4	3.8	R/O	15.8	White
6	Acc. 29	15.9	16.5	8.3	3.1	O	15.9	White

This result agreed with the finding of Alan et al. (2004). Three days after culturing flowers opened, and ovary got swelled (fig. 1c). Anthers started shriveling, and no dehiscence was observed in all media which confirmed its parthenogenesis origin. The apomictic testa (fig. 1e) formed in 43 days

old flower bud culture was neither correlated with embryo induction nor survival rate.

Effect of B5 media composition

Three combinations of auxin *viz*., 2,4-D, NAA and cytokinin *viz*., BA, 2-iP in B5 medium were used to study the multiplier onion effect on *in vitro* gynogenesis.

In HM1 medium, embryos got induced in four genotypes except Acc. 9 and Acc. 1. Per cent embryo induction ranged from 0.00-0.94 with Acc. 2 recordings the highest per cent embryo induction of 0.94 followed by Acc. 29 (0.81), Acc. 15 (0.74) and Acc. (0.44). Per cent callus induction ranged from 0.00-35.90 with Acc. 1 recording the highest per cent callus induction of 35.90 which were

Table 2. The B5 and MS medium with different composition of supplements

S. No	Haploid Medium	Medium	2,4-D	BA	NAA	2-iP	Putrescine	Spermidine	Sucrose
			(mg/l)				(mM)		(%)
1	HM1	B5	2	2	-	-	-	-	7.5
2	HM2	B5	-	-	1	1	-	-	7.5
3	HM3	B5	-	1	1	-	-	-	7.5
4	HM4	MS	2	2	-	-	-	-	10
5	HM5	MS	-	-	2	2	-	-	7.5
6	HM6	MS	2	2	-	-	2	0.1(after 14 days)	7.5

succeeded by Acc. 2 (12.26) and Acc. 29 (7.32). Three genotypes (Acc. 4, Acc. 9 and Acc. 15) did not record any callus formation. Somatic organogenesis was recorded only in Acc. 9 (0.41 %) and Acc. 15 (0.37 %). Plumpy bud was recorded only in Acc. 1 with 7.69 per cent induction. Embryo per cent recorded by Geoffriau *et al*. (1997) and Kher *et al.* (2018) in this HM1 media was higher than the present study.

Table 3. Pooled analysis of genotypes

Genotypes	mean %		
	Embryo	Callus	Plumpy
Acc. 4	0.76	0.06	0
Acc. 9	0	0	0
Acc. 15	0.48	0	0
Acc. 1	0.13	5.98	3.37
Acc. 2	0.17	2.04	0
Acc. 29	0.14	8.89	0.88

In HM2 medium, per cent of embryo induction in Acc. 4 was 0.46 and other genotypes did not recorded any embryo. But the use of HM2 composition in this study was reported to have higher embryo induction in this studies then by Michalik *et al*. (2000), Fayos *et al*. (2015) and Mathapati *et al*. (2018). Moreover, no callus, somatic organogenesis and plumpy bud were observed.

In the HM3 medium, the embryo was induced only in Acc. 15 with a per cent embryo induction of 0.40. Somatic organogenesis and plumpy bud were observed only in Acc. 1 with 7.5 % and 12.5 % respectively. Campion *et al*. (1992) observed HM3 had embryo induction per cent ranged from 0.258-0.40 in short day onion flower culture.

Effect of MS media composition

Two combinations of auxins (2,4-D, NAA) and cytokinin (BA, 2-iP) alone and supplemented polyamines *viz.,* putrescine, spermidine with 2,4-D and BA as one combination with MS as a basal medium was studied.

In HM4 medium, embryos were induced only in Acc. 4 and Acc. 15. Per cent embryo induction ranged from 0.00-0.36 with the highest embryo induction per cent recorded in Acc. 4 and Acc. 15

Table 4. Number of gynogenic shoots

Media/Acc.	HM 1	HM 2	HM 3	HM 4	HM 5	HM 6	Total
Acc. 4	3 (0.44)	1 (0.46)	0	3 (0.36)	1 (0.77)	2 (0.75)	10
Explant	686	217	287	836	261	267	2554
Acc. 9	0	0	0	0	0	0	0
Explant	737	212	387	281	292	239	2148
Acc. 15	4 (0.74)	0	1 (0.40)	1 (0.31)	0	4 (1.41)	10
Explant	541	174	250	321	200	284	1770
Acc. 1	0	0	0	0	2 (0.84)	0	2
Explant	392	188	403	392	239	314	1928
Acc. 2	1 (0.94)	0	0	0	0	0	1
Explant	106	272	376	462	289	323	1828
Acc. 29	1 (0.81)	0	0	0	0	0	1
Explant	123	198	290	352	405	410	1778
Total	9	1	1	4	3	6	24
Explant	2585	1261	1993	2644	1686	1837	12006

Frequency of embryo per cent represented in parentheses.

as 0.36 and 0.31 respectively. Per cent of callus induction ranged from 0.00-14.29 with Acc. 29 recorded the highest per cent callus induction of 14.29 followed by Acc. 4 (0.36). Other genotype does not respond to callus formation. The per cent of somatic organogenesis ranged from 0.00-17.90 with the highest per cent in Acc. 1 was 17.90 followed by Acc. 4 (0.72). No response was noticed in other genotypes.

Table 5. Comparison of stomata dimension

Parameters	Diploid stomata length (μM)	Diploid stomata width (μM)	Haploid stomata length (μM)	Haploid stomata width (μM)
Mean	22.21	14.76	15.84	8.29
Range	25.13-20.07	17.88-12.29	18.79-12.18	12.41-5.13
Standard error	± 0.36	± 0.372	± 0.4	± 0.39

This HM4 media composition was followed by Campion *et al*. (1992), Jakse *et al*. (1996), Geoffriau *et al*. (1997) and Anandhan *et al.* (2014). Muren (1989) was very first research in onion gynogenesis done with this media composition along with 10 per cent sucrose, but increase in sucrose concentration does not alter the embryo induction (Mahapati *et al.* 2018).

In HM5, per cent of embryo induction ranged from 0.00-0.84 with highest per cent embryo induction in Acc. 1 (0.84) followed by Acc. 4 (0.77). No embryo inductions in other genotypes were observed. No callus, somatic organogenesis and plumpy bud induction were recorded. The embryo per cent ranged from 0 to 1.2 reported by Michalik *et al*. (2000) in HM5 composition was less than the present study.

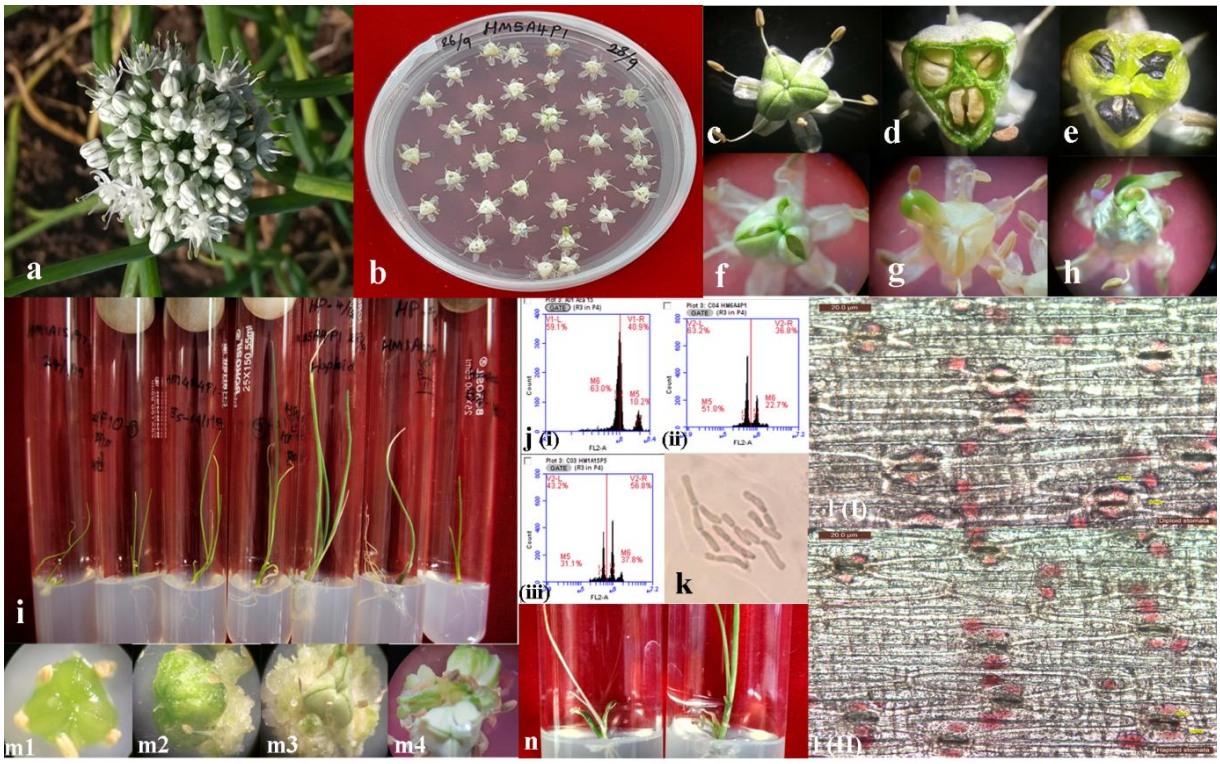

Figure 1. Haploid induction *via* gynogenesis. (a) Unopened flower buds three days before anthesis. (b) Flower buds inoculated in medium. (c) Ovary swelling and stigma enlargement. (d) Immature ovule. (e) Matured apomictic seed. (f) Rupturing of ovary wall. (g,h) Gynogenic embryo emergence. (i) Haploid plants. (j) Flow cytometry analysis of (i) control (diploid), (ii) haploid, (iii) mixoploid. (k) Cell cytology analysis. (l) Stomata dimension of (I) diploid, (II) haploid. (m) Abnormal growth comprises (1) hyperhydric bud, (2) plumpy, (3) callus, (4) somatic organogenesis. (n) Comparison of albino and normal plant

In HM6, the embryos were induced only in Acc. 4 and Acc. 15. Per cent of embryo induction ranged from 0.00-1.41 with the highest per cent of recording in Acc 15 of 1.41 followed by Acc. 4 (0.75). The callus and plumpy formation were recorded only in Acc. 29 with per cent of 13.71 and 2.44 respectively. Somatic organogenesis was recorded only in Acc. 4 with per cent of 1.12. This result was contrary to Martinez *et al.* (2000) who reported polyamines induced a significant effect on embryo formation and maturation.

Effect of genotype

Based on a pooled analysis of genotypes (table 3), the responsiveness of the genotypes towards per cent of embryo induction ranged from 0.00-0.76 with Acc. 4 recorded highest embryo formation per cent of 0.76. Acc. 9 did not respond to embryo induction in all media. The highest callus formation per cent observed in Acc. 29 was 8.89 and per cent of high plumpy bud recorded in Acc. 1 with 3.37. Acc. 9 and Acc. 15 did not record callus and plumpy bud formation in any of the medium.

Days to response

Time taken for the emergence of the embryo is an important factor for selection of media. A number of days taken to fastest response ranged from 46 to 60 days in HM5 followed by 64 to 81 days in HM 1, 72-105 days in HM6 and 78-135 days in HM4. The number of days to embryo formation in HM2 and HM3 was 83 and 66 days respectively.

Early embryo induction in 46 days obtained in Acc.1 and lateness in Acc. 29 (79 days). The embryo induction range of 46-54 days obtained in Acc. 1 followed by 64-105 days in Acc.15 and 60-135 days in Acc. 4. The days to response was on agreement with the finding of Sulistyaningsih *et al*. (2006) who obtained embryos in 40 days old flower culture in *A. cepa* var *aggregatum*.

Efficiency of gynogenesis

In B5 medium, 11 plantlets were induced, the induction rate was 0.21 per cent with a plant survival of 54.54 per cent. The average number of days taken for induction was 71 days. In MS medium, 13 plantlets were induced with an induction frequency of 0.34 per cent with a survival of 38.46 per cent. The average number of days taken for plantlet induction was 65 days.

In Acc. 15, two plantlets per single responsive flower bud were obtained from HM1 medium produced mixoploid and haploid, which was confirmed through flow cytometry analysis. Alan *et al*. (2004) reported six plantlets produced from a flower bud in YIX stock E but in the present research, not all the six ovules in the responsive bud were producing plantlets.

Albino was a common phenomenon in haploid induction studies (Anandhan *et al*. 2014). The per cent of albino recorded in B5 and MS medium were 18.9 (2 plants) and 23.1 (3 plants) respectively.

The genetic architecture and media composition were the prime factors for determining the gynogenic potential. In this research, low gynogenesis (table 4) recorded might be due to high heterozygous nature of cross-pollinated genotypes. This result was in agreement with Michalik *et al.* (2000) and Geoffriau *et al.* (1997) but contrast with studies of Kher *et al.* (2018) who observed that open-pollinated varieties (OPV) and hybrids were more amenable to gynogenesis.

Analysis of ploidy level

Out of 11 plantlets obtained on three media compositions on B5 medium, 6 (55%) plants survived. Out of 6 plants, 5 (83%) plants were haploid, 1 (17%) plant was mixoploid. Haploid and mixoploids were observed only in HM1 medium.

On MS medium, out of 13 plantlets, 5 (39%) plants survived, and all 5 (100%) were haploid. Out of 5 plants, 3 (60%) plants were obtained in HM4, 1 (20%) plant from HM5 and 1 (20%) plant from HM6.

The plantlets obtained from explant were confirmed under flow cytometry analysis, again subjected through cell cytology and stomata dimension test (table 5).

The survived plantlets from B5 and MS medium showed 10 (90.9%) were haploid and 1 (9.1%) was mixoploid. No tetraploid and spontaneous diploids were recorded in this study. This was contrast with the experiment of Alan *et al*. (2007) who obtained 73.2 % haploid, 13.5 % diploid, 10.3% mixoploid, 0.6 % triploid and 2.5 % tetraploid.

CONCLUSION

Inbred development has more advantage in hybrid development, mapping population and genetic studies especially in onion. In the present investigation, media composition was optimized in short day multiplier onion, whereas HM1 medium was found to be efficient than other media combinations. In genotype, Acc. 4 recorded higher per cent of gynogenic potential than other genotypes. This finding will pave the way for further research in the multiplier onion heterosis breeding program.

ACKNOWLEDGEMENT

This research was supported by a grant (code AS-046) from the Tamil Nadu State Council for Science and Technology, Chennai, India. Authors wish to thank Dr. S.Anandhan and ICAR-Directorate of Onion and Garlic Research for supporting this research.

REFERENCES

Anandhan S, Chavan A A, Gopal J, Mote S R, Shelke P V and Lawande K E. 2014. Variation in gynogenic potential for haploid induction in Indian short-day onions. Indian Journal of Genetics and Plant Breeding 74(4): 526–8.

Alan, A.R, Brants, A., Cobb, E.D., Goldschmied, P.A, Mutschler, M.A. and Earle, E.D. 2004. Fecund gynogenic lines from onion (*Allium cepa* L.) breeding materials.

Alan, A.R, W. Lim, M.A. Mutschler, and E.D. Earle. 2007. Comple- mentary strategies for ploidy manipulations in gynogenic onion (*Allium cepa* L.). Plant Sci. 173:25–31.

Abdelrahman, M, Y. Sawada, R. Nakabayashi, S. Sato, H. Hirakawa, M. El-Sayed, M. Y. Hirai, K. Saito, N. Yamauchi and M. Shigyo. 2015. Integrating transcriptome and target metabolome variability in doubled haploids of Allium cepa for abiotic stress protection. Mol. Breed. 35: 195.

Anonymous 2018 Month-wise export of fresh onion, 2018. https://www.indiastat.com/table/foreign-trade/12/horticulture-export/450234/1126998/data.aspx

Campion, B., Azzimonti, M.T., Vicini, E., Schiayi, M. and Falavigna, A. 1992. Advances in haploid induction in onion (*Allium cepa* L.) through in vitro gynogenesis. Pl.

Fayos O, Valle´s MP, Garce´s-Claver A, Mallor C, Castillo AM. 2015. Doubled haploid production from Spanish onion (*Allium cepa* L.) germplasm: embryogenesis induction, plant regeneration and chromosome doubling. *Front Plant Sci.,* 6:384

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations - FAO Statistical Database.

Germana MA, Chiancone B. 2001. Gynogenetic haploids of Citrus after in vitro pollination with triploid pollen grains. *Plant Cell Tissue Organ Cult* **66(1)**: 59–66.

Geoffriau E, Kahane R and Rancillac M. 1997. Variation of gynogenesis ability in onion (*Allium cepa* L.). *Euphytica* **94(1)**: 37–44.

Keller, J, 1990. Culture of unpollinated ovules, ovaries and flower buds in some species of the genus Allium and haploid induction via gynogenesis in onion (*Allium cepa* L.). *Euphytica* **47**, 241–247

Khar, A, Kumar, A, Islam, S, Kumar, A, & Agarwal, A. (2018). Genotypic response towards haploid induction in short day tropical Indian onion (*Allium cepa*). Indian Journal of Agricultural Sciences.

Martinez, L.E, Augero, C.B, Lopez, M.E. and Galmarini, C.R. 2000. Improvement of *in vitro* gynogenesis induction in onion (*Allium cepa* L.) using polyamines. Pl. Sci.,156:221-226

Muren R C. 1989. Haploid plant induction from unpollinated ovaries in onion. Hort Science 24(5): 833–4.

Michalik B, Adamus A and Nowak E. 2000. Gynogenesis in polish onion cultivars. Journal of Plant Physiology 156(2): 211–6.

Manjunathagowda D C, Bomble R Y and Vijay T K. 2015. Prosperity of Onions in India. Popular Kheti Volume -3, Issue-3 (July-September).

Mathapati, G. B, Kalia, P, Islam, S, Saini, N, Kumar, A, & Khar, A. 2018. Influence of Culture Media and Their Compositions on Haploid Induction in Indian Short Day Onion. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.

Roy S. 1989. Differentiation and anther culture of *Allium cepa*. Horticulture Germplasm. Cultivated and Wild Intl Symp 2:267–272 Acad. Publ., Beijing

Sulistyaningsih E, Aoyagi Y and Tashiro Y. 2006. Flower bud culture of shallot (*Allium cepa* L. *Aggregatum* group) with cytogenetic analysis of resulting gynogenic plants and somaclones. Plant Cell Tissue and Organ Culture 86(2): 249–55