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Genotypic response towards haploid induction in short day tropical Indian onion (*Allium cepa*)

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ABSTRACT

Doubled haploid technology is advantageous for a cross pollinated crop with high inbreeding depression like onion that takes 12 years to develop inbreds through conventional breeding. Inbred development through haploid technology takes only 1 to 2 years and will be a boon for heterosis breeding programme. Two media (HAP02, HAP04) and seven genotypes were assessed for their response towards haploid induction. HAP04 was significantly superior to HAP02 for embryo induction. Significant differences in media towards callus formation and significant differences in media and genotypes towards embryo induction were observed. Open pollinated varieties and hybrids were more responsive towards embryo induction followed by exotic and landrace, whereas callus induction was more in landrace. Days for embryo induction were lower in PWF (65-83), whereas RCL was the best responding genotype (2.22 shoots/plant) for multiple shoot induction. About 33% plants survived and haploids, spontaneous diploids and tetraploids were obtained. Gamborg's B5 medium supplemented with 2,4-D and BA @ 2 mg/l each was the best media. This study will form a basis for inbred development to accelerate hybrid breeding programme in short day tropical Indian onion.

Key words: *Allium cepa*, Flow cytometry, Gynogenesis, Haploid, Tropical onion

Hybrid onions are preferred by farmers because of their uniformity in colour, shape, size and maturity with the added benefit of higher productivity. India contributes to 17% of the total world production and occupies 19% of the gross cultivated area. Compared to countries with higher productivity, i.e. Republic of Korea (66.5 t/ha), Austria (64.1 t/ha), USA (55.9 t/ha) etc, India occupies 88th position with a productivity of 16.1 t/ha (FAOSTAT 2014). India, being a tropical country, onion is produced under short day conditions whereas countries with high productivity grow onions under long day conditions. Further, maximum area (>90%) in US, Europe and other countries where productivity is high are under hybrids which are high yielders. Whereas in India, open pollinated varieties (OPVs) are grown which are non-uniform and susceptible to major insect pests and diseases.

The primary objective for hybrid development is inbred availability. Availability of inbreds is difficult since onion suffers from severe inbreeding depression and being a biennial crop, takes around 10-12 years towards inbred development (Shigyo and Kik 2008). Despite 60 years of onion research in India, hybrids are not available to the onion farmers although hybrids are reported to be high yielders than the OPVs under tropical conditions (Singh and Bhonde 2011, Nunes *et al*. 2014). Hence, it is profitable to develop inbreds, at a faster pace, for hybrid development.

Non-conventional techniques, viz. *in-vitro* gynogenesis, androgenesis or microspore culture are helpful for accelerated inbred development. In onion, haploid development through *in-vitro* gynogenesis is being practiced since 1989 (Muren 1989). Studies on the effect of medium compositions, donor material, age of donor material, genotype responsiveness, polyamines etc for haploid development (Fayos *et al*. 2015) have been published. But majority of the research studies have been conducted in long day onion. It is a very well known fact that genotype plays a very important role towards haploid induction and subsequent doubled haploid formation (Geoffriau *et al*. 1997, Michalik *et al*. 2000). In India, there are only two reports on haploid induction in short day onion (NRCOG 2007, Anandhan *et al*. 2014) and more research studies need to be undertaken so that a standardized protocol for haploid induction is established. The main aim of this study was to identify the best medium for haploid induction and to study the response of genotypes towards haploid induction.

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MATERIALS AND METHODS

Four open pollinated varieties (Pusa Red, Pusa Madhavi, Hisar-3, Pusa White Flat-PWF), one landrace (Balwan Pyaz-BPZ), one exotic variety (AVON1101-AVON) and one exotic hybrid (Red Coral-RCL) were used in the study. Bulbs were sown in first fortnight of November under open field conditions at IARI, New Delhi. Unopened flower buds (3.5 mm to 4.5 mm) were used for inoculation when 15-25% flower buds in an umbel were open. Surface sterilization consisted of immersion of buds in 0.3% Tilt alongwith 2 to 3 drops of Tween 20 for 10 min followed by five washings with tap water. Flower buds were then treated with 70% ethanol for 2 min, rinsed three times and then treated with 4% sodium hypochlorite for 4 min and rinsed 3 to 4 times under aseptic conditions. Basal media consisted of Gamborg's B5 media (Gamborg *et al*. 1968) supplemented with 75 g/l sucrose. Two B5 based media, i.e. HAP02 (2,4-D @ 1 mg/l + BA @ 2 mg/l) and HAP04 (2,4-D @ 2 mg/l + BA @ 2 mg/l) were used. All the media were adjusted to pH 5.8 with 1N HCl or 1N NaOH, autoclaved and poured into 90 × 20 mm petri-dishes and culture conditions were maintained at 25±2ºC with 16/8h (light/dark) light period with a light intensity of 50μ^2/Ems. For multiple shoot induction, Gamborg's B5 medium containing sucrose @ 2% supplemented with NAA (1.0 mg/l) + BA (1.0 mg/l) (OMS1) and NAA (1.0 mg/l) + BA (2.0 mg/l) (OMS2) + activated charcoal (0.5 g/l) were tried. Young leaves of *in vitro* induced onion plants were analyzed for their ploidy status using CyFlow®Cube 6 using *in-vitro* onion seedlings of known ploidy as control. Plants were acclimatized by transferring them to jam bottles containing sterile perlite saturated with half- strength MS medium for 1 week without aeration and additional one week by opening the lid (Method 1). In second method, plants were planted in multicell trays containing sterile soilrite mix and covered with plastic sheet, maintaining 100% humidity, for a week and then the sheet was opened slowly over a period of one week.

The experiment was laid out in completely randomized design. Petri-dish was taken as an experimental unit and each petri-dish consisted of 25 flower buds and the number of repetitions ranged from 9 to 17. The data so collected was arc sin transformed and analyzed using PROC GLM in SAS 9.3. Data were subjected to analysis of variance (ANOVA) and to test the significance of the treatments means, Duncan's Multiple Range Test (DMRT) was employed. Interactions between media, treatment and replications were analyzed to ascertain the best media and the response of genotype towards haploid induction.

RESULTS AND DISCUSSION

Effect of medium composition on callus induction

A total of 3240 flower buds were used for gynogenesis induction. In medium HAP02, per cent callus induction ranged from 0.00-4.43% with BPZ recording highest per cent callus induction (4.43) which was significantly at par with RCL (2.24%), Pusa Madhavi (1.17%) and Pusa Red (0.94%). Three genotypes (PWF, Hisar 3, AVON) did not record any callus formation. In HAP04, highest callus induction was also reported in BPZ (4.92%) and was significantly at par with RCL (2.97%) and significantly more than Pusa Madhavi (1.08%). Four genotypes, viz. Pusa Red, PWF, Hisar 3 and AVON did not record any callus formation (Table 1). Based on pooled analysis, responsiveness of genotypes towards per cent callus induction ranged from 0.00 – 4.66. Highest per cent callus was observed in BPZ (4.66) followed by RCL (2.58), Pusa Madhavi (1.12) and Pusa Red (0.45). Callus was absent in PWF, Hisar 3 and AVON (Table 2). Significant differences for per cent callus induction were observed among the genotypes. Based on the ANOVA, it was observed that medium was not significant for callus induction (Pr>F 0.89) but genotypes differed significantly in per cent callus induction (Pr>F 0.0023) (Table 3). It was also observed that medium × treatment, treatment × replication and medium × replications interactions were non-significant. On DMRT analysis, both mediums, viz. HAP02 (mean=1.23) and HAP04 (mean=1.15) were at par with each other. Callus induction at the base of flower is a common phenomena and has been reported by Bohanec and Jakše (1999) and Sulistyaningsih *et al*. (2006). Bohanec and Jakše (1999) observed callus (0.7-45.0%) in all the accessions under study in contrast to our studies where calli range was 0.0 - 4.9% and was present only in three genotypes. Bohance and Jakše (1999) observed that formation of basal callus was not directly correlated to lower embryo induction which corroborated agreement with our findings. Hence, potential of a medium and the genotype cannot be analysed by its callus forming ability. Flower buds which form callus are of no use and need to be discarded but Sulistyaningsih *et*

Table 1 Response of genotypes towards callus formation and embryo induction in individual medium

Treatment	Status	Replications	Medium	Mean callus (%)	Mean embryos (%)
Pusa Red	OPV	16	HAP02	0.94^{ab}	1.34^{ab}
Pusa Madhavi	OPV	11	HAP02	1.17^{ab}	4.42^{ab}
PWF	OPV	9	HAP02	0.00^{b}	5.74^{a}
Hisar 3	OPV	11	HAP02	0.00^{b}	3.52^{ab}
BPZ	LR	10	HAP02	4.43^{a}	1.29^{ab}
AVON	EV	13	HAP02	0.00^{b}	0.00^{b}
RCL	EH	14	HAP02	2.24^{ab}	1.84^{ab}
Pusa Red	OPV	17	HAP04	0.00^{b}	3.61^{ab}
Pusa Madhavi	OPV	12	HAP04	1.08^{b}	6.05^{ab}
PWF	OPV	9	HAP04	0.00^{b}	6.84^{a}
Hisar 3	OPV	11	HAP04	0.00^{b}	6.37^{ab}
BPZ	LR	9	HAP04	4.92^{a}	0.00^{b}
AVON	EV	11	HAP04	0.00^{b}	1.67^{ab}
RCL	EH	12	HAP04	2.97^{ab}	6.92^{a}

OPV- Open pollinated variety, LR-Landrace, EV-Exotic variety, EH – Exotic hybrid

Table 2 Pooled analysis of genotypes response towards callus formation and embryo induction

Treatment	Replications	Mean	
		Callus (%)	Embryos (%)
Pusa Red	33	0.45[b]	2.51[ab]
Pusa Madhavi	23	1.12[b]	5.27[a]
PWF	18	0.00[b]	6.29[a]
Hisar 3	22	0.00[b]	4.95[a]
BPZ	19	4.66[a]	0.68[b]
AVON	24	0.00[b]	0.77[b]
RCL	26	2.58[ab]	4.19[ab]

Table 3 ANOVA for medium, treatment and replication interactions for callus induction in onion genotypes

Source	DF	Type I SS	Mean square	F value	Pr>F
Med (Medium)	1	0.303	0.303	0.02	0.8923
Trt (Treatment)	6	387.061	64.510	3.93	0.0023
Rep (Replication)	16	282.474	17.655	1.08	0.3981
Med*Trt	6	10.288	1.715	0.10	0.9956
Trt*Rep	63	1062.150	16.859	1.03	0.4599
Med*Rep	15	185.755	12.384	0.75	0.7193

al. (2006) used the callus derived plants which were either diploid, tetraploid or mixoploid in nature.

Effect of medium composition on embryo induction

In HAP02, embryos were induced in six genotypes except AVON. Per cent embryo induction ranged from 0.00-5.74 in HAP02 and 0.00-6.92 in HAP04. In HAP02, highest per cent embryo induction was in PWF (5.74) and was at par with all the genotypes except AVON (Table 1). In HAP04, highest per cent embryo induction was in RCL (6.92) which was at par with all the varieties except BPZ. Rate of embryo induction was higher than reported in Indian varieties (Anandhan *et al*. 2014), Spanish germplasm (Fayos *et al*. 2015), Polish cultivars (Michalik *et al*. 2000) but less than some of the American inbred and F1 lines (Bohanec and Jakše 1999). Embryos were not induced in AVON (0.00) in HAP02 and BPZ in HAP04. Recalcitrant varieties towards embryo induction have also been reported by other researchers (Geoffriau *et al*. 1997, Bohanec and Jakše 1999, Michalik *et al*. 2000). Pooled analysis recorded that highest per cent embryo induction was in PWF (6.29) and was significantly at par with Pusa Madhavi (5.27), Hisar 3 (4.95), RCL (4.19), Pusa Red (2.51) and significantly superior than BPZ (0.68) and AVON (0.77) (Table 2). PWF, Pusa Madhavi, Hisar 3 and Pusa Red are OPVs and they were at par with exotic hybrid RCL (4.19) but significantly superior than landrace (BPZ) and exotic variety (AVON). Geoffriau et al. (1997) proposed that genetic structure of varieties explains gynogenesis ability and genotype of a donor plant has crucial influence on haploid induction ability (Bohanec & Jakše, 1999; Michalik *et al.*, 2000). In our experiments, OPVs and hybrids were more responsive followed by exotic material (AVON) and landrace (BPZ) which is in contrast to other reports (Geoffriau *et al*. 1997, Bohanec and Jakše 1999, Michalik *et al*. 2000) who observed that hybrids were more responsive followed by inbreds/synthetics and open pollinated varieties. Probable reason may be that the OPVs used in the pre cent experiment are propagated through controlled pollination since they are used for varietal maintenance, which may have led to homogeneity in the material. On ANOVA analysis, it was observed that medium (Pr>F 0.0248) and treatment (Pr>F 0.0172) were significant but replications and medium × treatments, treatments × replications and medium × replications interactions were not significant (Table 4). DMRT analysis observed that medium HAP04 (n=81, mean=4.53) was statistically significantly superior than HAP02 (n=84, mean=2.37) in embryo induction. Medium composition (Jakše *et al*. 1996, Michalik *et al*. 2000) has been found to be an important criterion towards gynogenesis induction and Fayos *et al*. (2015) were able to induce 2-3 times higher embryogenesis percentage using the two-step protocol described by Michalik *et al*. (2000). Hence, medium plays an important role in increasing the gynogenesis potential. Almost all the haploid induction studies in onion are based on the combination of 2,4-D and BA @ 2 mg/l each but most of the authors use BDS medium for induction studies now. This experiment proves that this combination is also good for short day onion also.

Days taken to embryo induction

Average number of days ranged from 57-171 days in HAP02 and 76-114 days in HAP04. Fastest induction (45 days) occurred in Pusa Red whereas less range of days (75-85) were taken by RCL for embryo induction in HAP02. In HAP04, fastest induction was observed in RCL (60 days) and most number of days were taken by Hisar 3 (223 days). The results corroborated with the induction days reported by Michalik *et al*. (2000) and Geoffriau *et al*. (1997) but more than reported (40-98 days) in short day onion (Anandhan *et al*. 2014). PWF took less number of days (65-83) for embryo induction which was in agreement with Jakše *et al*. (1996) who observed that cultivars with high regeneration capacity regenerated in shorter range of days, whereas regenerants with low frequency took more

Table 4 ANOVA for medium, treatment and replication interactions for embryo induction in onion genotypes

Source	DF	Type I SS	Mean Square	F value	Pr>F
Med (Medium)	1	192.254	192.254	5.32	0.0248
Trt (Treatment)	6	616.787	102.798	2.84	0.0172
Rep (Replication)	16	487.825	30.489	0.84	0.6336
Med*Trt	6	118.895	19.816	0.55	0.7696
Trt*Rep	63	2692.966	42.745	1.18	0.2618
Med*Rep	15	725.396	48.360	1.34	0.2115

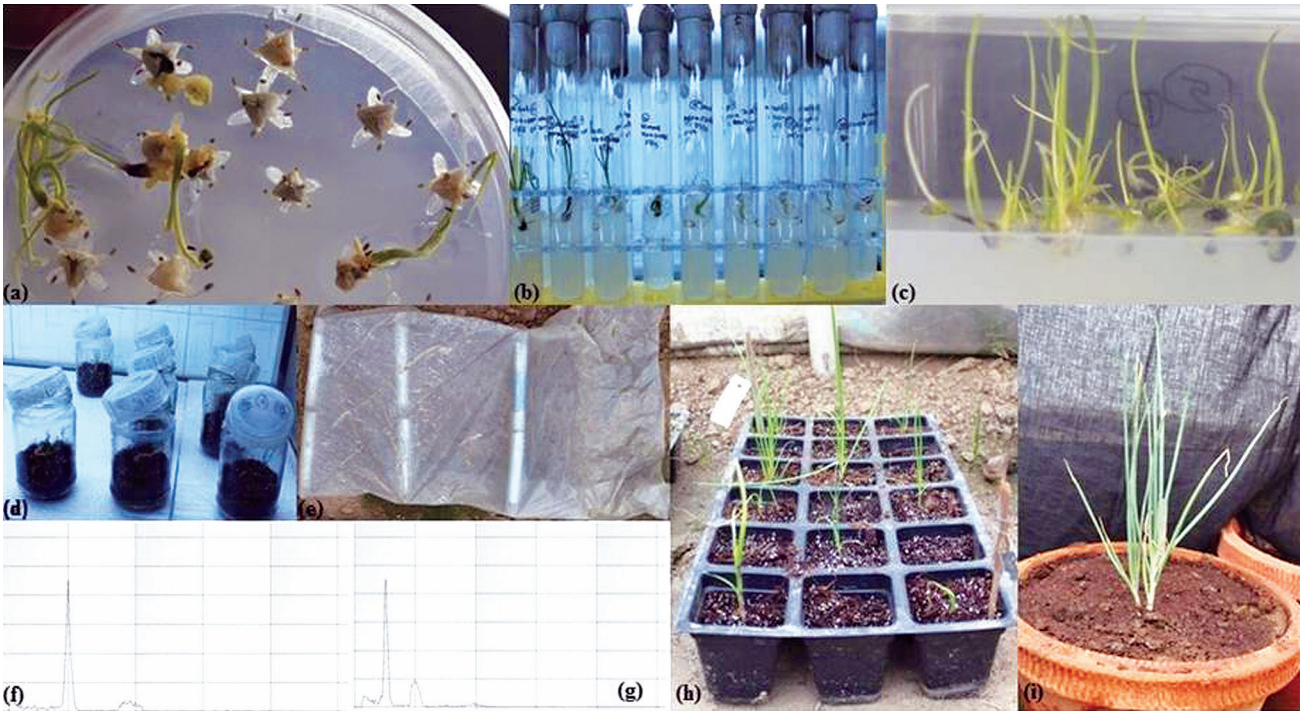

Fig 1 (a) Plantlet induction from flower buds cv. Red Coral (b) Culturing induced plantlets for root and shoot formation (c) Multiple shoot induction in regenerated plantlets (d) Acclimatization in jam bottles (e) Acclimatization under polythene tunnel (f) Flow cytometry analysis of a control (diploid) plantlet (g) Flow cytometry of a haploid plantlet (h) Hardened plants in multicell tray (i) Plants shifted to pots for growth and bulb development.

number of days.

Multiple shoot induction

Only medium OMS2 responded to multiple shoot induction in most of the genotypes. RCL was the best responding genotype (2.22 shoots/plant) followed by Pusa Madhavi (2.00), Hisar 3 (1.78), PWF (1.10) and Pusa Red (0.14). Induction of multiple shoots in regenerated plants has an added advantage that the mother plant, with known ploidy level, can be used as a source for increasing the plantlets. Geoffriau *et al*. (1997) observed that most of the clones expressed same ploidy level as the original plant after one cycle of micropropagation. A standardized genotype independent protocol for multiple shoot induction will aid in increasing the number of plants for doubled haploidization and *ex vitro* studies.

Analysis of regenerated plants through flow cytometry

Out of the 17 plants, eleven (65%) haploids, four (23%) spontaneous diploids and two (12%) tetraploids were obtained. Geoffriau *et al*. (1997) observed 79.6% haploids, 13.1% diploids and 1% tetraploids. Moreover, spontaneous diploids are advantageous since they can be used directly as inbreds but the homozygosity needs to be ascertained first by using molecular markers.

Acclimatization and transfer to ex vitro conditions

Thirty two plants were transferred to the glass jam bottles according to first method. Most of the plants died due to non-aerated conditions. Surviving plants were unable to withstand for additional week and died immediately after the bottles were opened. In second method, 52 plants were transferred and 17 (33%) plants were able to survive when transferred to the *ex vitro* conditions after 14 days of acclimatization. Acclimatization of plants for transfer to *ex vitro* conditions is more difficult in monocot, e.g. onion as compared to dicots. Low percentage of acclimated onion plants have also been reported by Fayos *et al.* (2015). A step by step guide for haploid induction studies in onion genotypes is depicted in Fig 1.

Majority of the haploid research studies are on long day cultivars, whereas tropical short day Indian onion has received less attention. Our aim was to evaluate media and genotypes amenable for haploid induction in Indian material and comparison with the exotic ones. Based on the results, it was observed that Indian OPVs and hybrids were more responsive than the exotic variety and landrace. Further, a multiplication media and method for acclimatization of plants before transfer to field conditions was also standardised. This protocol will form a stepping stone for doubled haploid induction, to be used as parents, for accelerated hybrid breeding programme in short day tropical onion.

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