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Callus induction and plant regeneration through unpollinated ovary culture in China aster (*Callistephus chinensis* L. Nees)

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Abstract
A protocol for successful callus induction and plant regeneration through unpollinated ovary culture in *Callistephus chinensis* L. Nees, has been described. Firstly, ovaries were initiated onto eight different media out of which B_5 medium augmented with BAP 2 mg/l + NAA 0.5 mg//l had the highest frequency of ovary response towards callus formation. Thereafter, three different bud stages were examined for their callus formation through ovary culture. The bud stage that took 10 days for anthesis was found to be the best stage for callus formation. Five different regeneration media were tested for shoot regeneration from callus with BAP 0.1 mg/l to 1mg/l and NAA level was kept constant at 0.01 mg/l. The highest induction and proliferation of indefinite buds was produced on MS + BAP 0.1 mg/l + NAA 0.01 mg/l. Thereafter, the shoots were elongated onto MS medium containing 2 mg/l BAP. Rooting was carried out on half-strength MS medium supplemented with IBA 1 mg/l. This protocol provides a useful foundation for further development of haploids through which homozygous lines can be developed.

Keywords: *Callistephus chinensis*, China aster, protocol, bud stage, callus induction, unpollinated ovary, culture, shoot, regeneration, elongation, proliferation, rooting

Introduction
China aster (*Callistephus chinensis* (L.) Nees) is a self-pollinated annual flower crop (North, 1979; Watts, 1980) belonging to the family Asteraceae. A single wild species *Callistephus chinensis* is responsible for development of present day China asters. It is known for its availability in a wide spectrum of colours and forms, long vase life, hardy nature and relatively easy to grow all-round the year. It has a got great functional utility in preparation of garlands, in bouquets as fillers and in exhibitions and also in flower arrangement. It is even popular as a bedding plant and also used in herbaceous borders in gardens. It is a great choice as a potted plant and the dwarf cultivars make a good edge.

Ovary as well as anther culture provides a chance for developing haploids and evaluating them for desirable traits which get masked due to presence of dominant alleles. Flower colour stabilization and disease resistance are some of the major problems in China aster. It was not until the works of Guha and Maheshwari (1964) [10] that made it possible to generate haploids through artificial means by *in vitro* culture of immature anthers of *Datura innoxia*. This also introduced the idea of obtaining haploids through ovaries and ovules which was described by San Noem (1976) [22] in barley. Plant geneticists and breeders were aware of the beneficiaries of pure lines and it was through overwhelming research works in development of haploids and double haploids, could they realize the potentialities of double haploid technology in speeding up the breeding cycle by achieving homozygosity in a single step through development of doubled haploids having the required traits.

Various genera of the Asteraceae family have proved to be incompetent regarding microspore embryogenesis. This is due to presence of somatic hairs that have a similar diameter as microspores which find their way through the screens used to separate debris from microspores (Ferrie and Caswell, 2011) [7]. Hence, ovary, ovule and anther culture were found to be alternatives for microspore culture. The present experiment was conducted standardize a protocol for regeneration of plants through ovary culture in China aster. Ovary culture is a preliminary step for development of haploids and this attempt is one of the first tissue culture study in China aster as per our knowledge.

Materials and Methods

Plant materials

China aster variety AAC-1 (Arabhavi Aster Collection-1) was selected for the experiment. The plants were grown under open conditions. The plants were nourished with recommended dose of fertilizers and every step was taken to minimize pest and disease incidence.

Preparation of induction media and induction of callus

Eight different media (Table1) having specific growth regulators were selected to determine the best induction medium for callus formation of *C. chinensis* through ovary culture. Each medium was supplemented with 30 g/l sucrose as a carbon source and the gelling agent used was Gelwell at 2.5 g/l. The pH was adjusted to 5.7-5.8. The media were sterilized using an autoclave at 121ºC and 15 lb pressure. All the media were kept and observed for a minimum period of three days for any contamination before they could be used for culture. Baby jar bottles as well as borosil test tubes with autoclavable polypropylene caps of 250 ml capacity and 100 ml respectively, were used as culture containers.

Unpollinated flower buds were collected from the donor plants five days before anthesis. They underwent a thorough washing under running tap water for 15 minutes. Thereafter, they were sterilized using 70% (v/v) ethanol solution for 35 seconds. This was followed by rinsing with distilled water for 3-4 times and subsequently, they were treated with 0.1% mercuric chloride for 5 minutes and a final rinse with sterile water for 5-6 times. All these treatments were carried out in sterile conditions inside a laminar air flow chamber except for the running tap water treatment. After surface sterilization, ovaries and anthers were excised from hermaphrodite and ray florets of the variety AAC-1. The excised ovaries were inoculated on different media supplemented with various combinations and concentrations of auxin and cytokinins (2,4-D, BAP, IAA, NAA and Kinetin) at the rate of four ovaries per test tube and ten ovaries per bottle. All the laboratory experiments were directed under well-defined condition of the culture room maintained at a temperature of 25 ± 2ºC. Fluorescent tubes (7200ºK) were used to provide a uniform light intensity (ca 1000 lux) over a light and dark cycle of 16 and 8 hours respectively. The frequency of ovaries responding to callus induction were determined 45 days after the ovaries were placed into culture. In order to determine the significance of the variables (p= 0.01 level), analysis of variance (one-way ANOVA) was used with three replicates. The significant difference between treatment means was determined using Duncan's multiple range test.

Determination of best bud stage for callus formation

The relationship between flower bud morphology, bud size and callus formation were studied after the best induction medium was determined. Three bud stages were selected (S_1- size: 0.5-0.75 cm and the tip of the buds did not show any tinge of color; S_2 - size: 0.75-1 cm and the tip of the buds exhibited a color tinge which was characteristic of the respective variety and S_3- Size: 1-1.5 cm and the unopened buds had ray florets which had entirely developed the color of the respective variety) and the best induction medium with the specific growth regulator concentration was used to determine the best bud stage. Ovaries were excised from the three bud stages and cultured onto the best induction medium and laboratory conditions were maintained as specified in the previous experiments. A completely randomized design was followed with eight replicates having ten ovaries inoculated per bottle.

Preparation of differentiation media and plant regeneration

Five different differentiation media labelled as D_1 - D_5 (Table 2) were tested. Four levels of BAP (0.1, 0.2, 0.5 and 1.0 mg/l) were tested with a constant level of NAA (0.01 mg/l). For all the five media, MS salts were combined with 30 g/l sucrose and 2.5 g/l gelwell. The pH was adjusted to 5.7-5.8. The media were sterilized using an autoclave at 121ºC and 15 lb pressure. Baby jar bottles with autoclavable polypropylene caps of 250 ml capacity were used as culture containers. Only the calli produced on the best induction medium were used for callus regeneration. The bottles were incubated in culture room maintained the specified laboratory conditions in the previous experiments. After 30 days, the regenerated shoots were excised and placed onto elongation medium having MS basic salts, pH 5.7-5.8, sucrose 30 g/l, gelwell 2.5 g/l and BAP 2 mg/l. Two to three subcultures were carried out till well developed shoots were seen. The experiment was carried out under completely randomly design with four replicates, having one bottle per replicate with four calli per bottle, for each differentiation medium.

Plant rooting

Healthy shoots that differentiated from green calli with 3 to four true leaves were excised from the culture and transferred to rooting medium. The rooting medium comprised of half-strength MS salts with sucrose 30 g/l, gelwell 2.5, pH 5.7-5.8 and supplemented with IBA 1 mg/l and the autoclaving was performed as previously described.

Results

Callus induction: The cultured ovaries were seen to bulge after ten days of culture. After 20 days of culture certain white to cream coloured calli were observed. As the calli continued to grow, the colour changed to yellow after 35 days of culture. After 50 days of culture, the calli exhibited three different morphologies. The first class comprised of greenish white, granular and compact calli, the second class had yellowish, translucent and watery callus and the third class exhibited reddish brown with friable soft calli. There was significant difference between the eight different media on which the ovaries were cultured. The highest percentage of callus induction from the ovaries was seen in Gamborg B_5 medium augmented with BAP 2 mg/l and NAA 0.5 mg/l approximately 3.5 times higher as compared to other media was seen whereas MS medium having BAP 2 mg/l, KN 2 mg/l and IAA 0.5 mg/l, had the lowest rate of callus initiation (Fig. 1) (Table 1). Early response of the cultured ovaries towards callus initiation was seen on Gamborg B_5 medium whereas the time taken by ovaries cultured onto C_m and Nitsch and Nitsch media were slow to respond. Calli of good sizes were produced from ovaries cultured onto Gamborg B_5 medium containing BAP 2 mg/l + NAA 0.5 mg/l which Poor calli sizes were noticed amid other media which didn't have much difference in their sizes (Table 2). The colour and growth pattern of the calli also varied. Green calli were produced from ovaries cultured onto MS, C, Nitsch and Nitsch and NLN media. Gamborg B5 medium and N6 medium yielded greenish white calli whereas brown calli were noticed in C_m and modified Ahmin and Vieth media (Fig. 3).

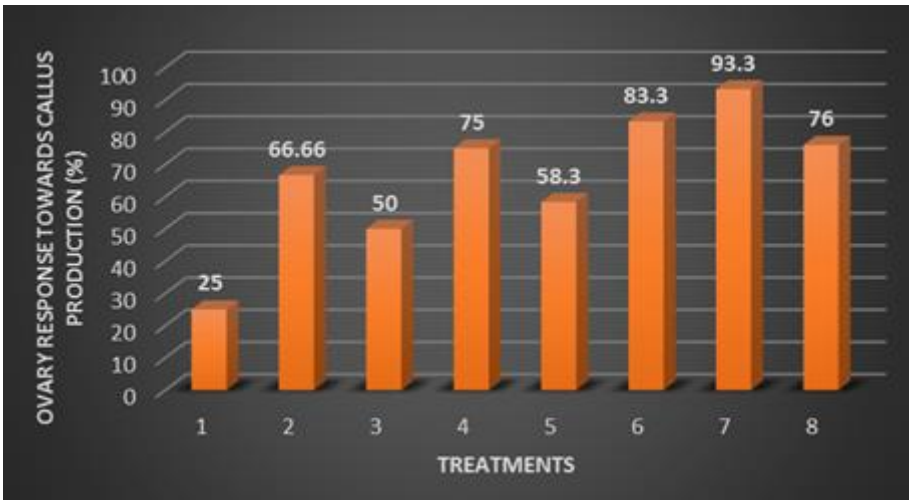

Fig 1: Effect of different media on callus production from ovaries of China aster cv. AAC-1

Determination of bud stage for callus formation

As Gamborg B_5 medium was found to be the best induction medium for callus formation, the ovaries excised from the three different bud stages *viz.* S_1, S_2, S_3, were cultured onto this basal medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l. The ovaries cultured from S_1 stage showed exceptionally higher response towards callus formation. This was followed by S_2 stage which also showed a slightly lower ovary response when compared to S_1 stage towards callus production (Table 3). S_3 stage set a lower trend towards callus production from the ovaries. The callus size showed considerable variations for the three bud stages. It was noteworthy that there was no significant difference in production of callus from ovaries cultured from S_1 stage and S_2 stage as both produced calli of similar sizes whereas ovaries cultured from S_3 stage exhibited calli size of significantly lower sizes as compared to the other two stages the ovaries cultured from all the three bud stages produced greenish white calli.

Shoot differentiation: Shoot initiation is one of the prime aspects for any tissue culture protocol and is highly dependent on the media composition as well as the growth regulator concentration. The shoot differentiation rate was seen to vary significantly across the treatments. Initially, greenish shoot buds appeared on the calli which later differentiated into shoots. Shoot differentiation was seen at around 25 days from the point wherein the calli were cultured for shoot regeneration. Calli that were yellowish or brown failed to develop the shoot buds while certain calli were also found to produce aerial roots. The per cent of shoot differentiation was found to be significantly higher for the treatment MS medium containing BAP 0.1 mg/l + NAA 0.01 mg/l (Fig. 2) (Table 4). The treatment MS medium having no growth regulators also showed the lowest shoot differentiation rate. The shoot length was seen to have its maximum length in MS medium containing BAP 0.1 mg/l + NAA 0.01 mg/l (Fig. 3) as well as BAP 1 mg/l + NAA 0.01 mg/l achieved its minimum length on MS medium that had no growth regulators within it.

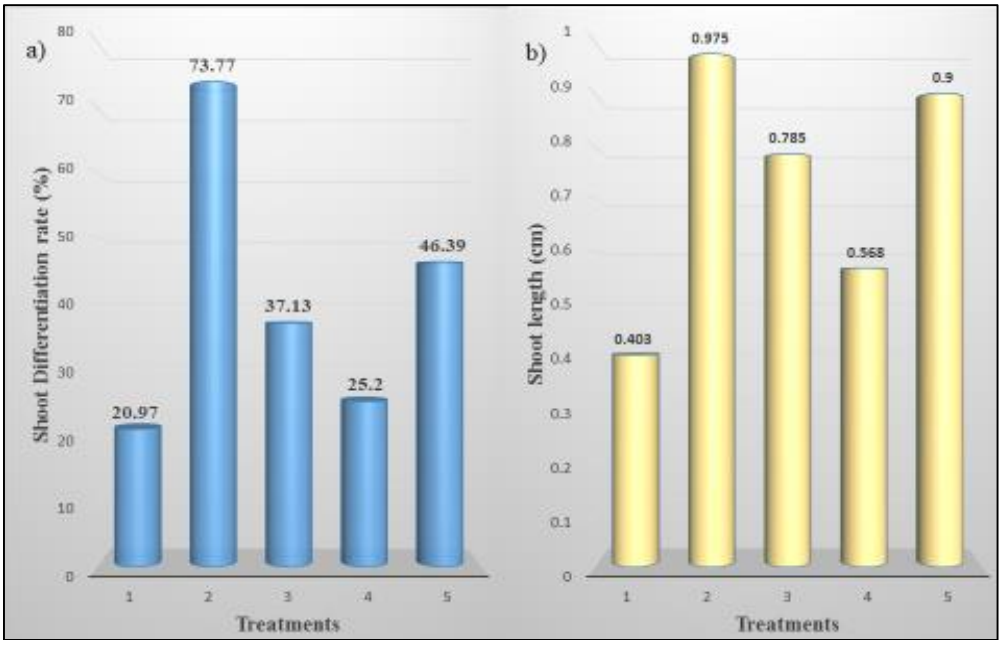

Fig 2: a) Effect of different media on shoot differentiation rate b) Effect of different media on shoot length

Discussion

Basal media type is one of the key components for regulating the induction and development of gynogenic haploids (Kumar *et al.*, 2020) [16]. It is also noteworthy that a single specific media cannot be considered suitable for all plant species. The medium composition and plant growth regulator

concentration play a vital role in callus formation and its differentiation (Jimenez, 2005) [13]. Callus induction occurs at an appropriate ratio of auxin and cytokinin (Rout and Das, 1997) [21]. In this investigation, the highest response of ovaries towards callus production was seen in the treatment Gamborg B_5 medium supplemented with BAP 2 mg/l and NAA 0.5 mg/l for the variety AAC-1. This showed that BAP along with NAA might have a significant role to play in callus induction (Obukosia *et al.*, 2005) [18]. At low concentrations, NAA, an auxin, is known to instigate cell development as well as enlargement. When cell enlargement occurs, the enzyme activity increases which in turn affects the cell wall plasticity and the generation of fresh cell wall materials (Cleland, 1971) [4]. Our results were parallel to the findings of Zhang *et al.* (2011) [29] who reported that Gamborg B_5 supplemented with KN 1 mg/l and 2,4-D 0.5 mg/l was the best medium for callus production from anthers of *Zantedeschia aethiopica*. The superior performance of Gamborg B_5 over MS medium was also seen during anther cultures of *Cyclamen persicum* (Ishizaka and Umematsu, 1993) [11] and *Lilium longiflorum* (Arzate-Fernandez *et al*., 1997) [1]. It has been postulated that ammonium concentrations at lower levels in Gamborg B_5 medium, in comparison to MS, would have been responsible for higher callus formation (Zhang *et al*., 2011) [29]. It was noticed that the callus formation from the pollens was augmented at a low concentration of ammonium ions and a higher concentration was found to be inhibitory for callus formation in rice anther culture (Chu *et al*., 1975) [3]. This was also confirmed with the retardation of growth of soya bean suspension cultures on treatment with a high concentration of ammonium ions (Gamborg *et al*., 1968) [8]. Nevertheless, low ammonium concentrations were also seen in other media like NLN and N_6, which were also utilised in our experiments which shows that a low ammonium level may not the lone reason for the exceptional performance of Gamborg B_5 medium. An analysis of the media composition shows that it has a higher level of the vitamin thiamine HCl (10 mg/l) when compared to other media. The energy production through carbohydrate metabolism utilises thiamine as an enzymatic cofactor which encodes its significance in energy management of the cells (Goyer, 2010) [9]. This may be responsible for the early initiation and large size of callus during the ovary culture of China aster.

The time taken for callus initiation from ovaries of China Aster cultured onto Gamborg B_5 medium containing BAP 2 mg/l and NAA 0.5 mg/l was found to be 20.26 days which was on par with the findings of Thaneshwari *et al*. (2018) [25] wherein the ovaries of *Tagetes erecta* treated at 25° C initiated callus on MS medium containing BAP 0.5 mg/l and NAA 0.1 mg/l after 21 days of culture. Comparable results were acquired by Khandkar *et al*. (2014) [14] wherein the callus initiation took 20-22 days when anthers of *Dendranthema grandiflora* were cultured onto MS medium containing BAP 2 mg/l and 2,4-D 1 mg/l. The findings of Wang *et al*. (2014) [26] were akin to our results in which they could obtain callus initiation from ovules of *Chrysanthemum morifolium* cultured onto MS medium supplemented with MS medium containing BAP 0.5 mg/l and NAA 0.1 mg/l in about 20 days of culture.

The anther response frequency of *Zantedeschia aethiopica*. towards callus production on Gamborg B5 medium with KN 1 mg/l and 2,4-D 0.5 mg/l as revealed by Zhang *et al*. (2011) [29] was found to be just 4.5per cent as compared to our experimental results which had an ovary response of 93.3 per cent towards callus production on the same basal medium supplemented with BAP 2 mg/l and NAA 0.5 mg/l. The experimental findings of Kumar *et al*. (2020) [15] showed that the ovules of *Tagetes erecta* when initiated onto enriched MS medium had 10.8 per cent response towards callus production which was much lower than our findings. The experiments conducted by Wang *et al*. (2014) [26] revealed that the response of ovules of *Chrysanthemum morifolium* cultured onto MS medium containing BAP 0.5 mg/l and NAA 0.1 mg/l was only 24.5 per cent in comparison with our results. The maximum callus size in our experiment was found to be 0.988 cm which was similar to the observations taken by Thaneshwari *et al*. (2018) [25] who obtained a maximum callus size of 1.19 cm from ovaries excised from buds of *Tagetes patula* pre-treated at 35ºC and cultured onto MS medium containing BAP 0.5 mg/l and NAA 0.1 mg/l. Our findings were also significantly higher than the anther derived callus size of 0.4-0.5 cm from N_6 medium with BAP 0.25 mg/l and NAA 0.06 mg/l as obtained during the experimental findings of Zhao *et al*. (2006) [30] in *Echinacea purpurea*.

The callus growth pattern played a significant role in determining the shoot regeneration capacity. Green and greenish white calli had a high affinity towards shoot regeneration whereas the calli that were yellowish white and brownish white seem to show poor or no regenerative capacity at all. These findings were confirmed with similar observations by Jia *et al*. (2014) [12] wherein the greenish calli had a high shoot regeneration capacity during the anther culture of baby primerose (*Primula forbesii*). Winarto *et al*. (2011) had analysed the regeneration capacity of anther derived calli of *Anthurium* which were of different colours. The analysis revealed that green to light green colour calli had a high regeneration capacity whereas reddish green callus showed poor shoot regeneration capacity. Thus, it can be suggested that the callus colour could be considered as a good determining criterion for selecting callus with good differentiation capacity in China aster plantlets.

One of the most critical factors governing the process of haploid induction is the developmental stage of female gametophyte as it plays a key role reconfiguring the pathway from gametophytic to sporophytic (Chen *et al*., 2011) [2]. One of the indicators of gametophytic development stage was found to be the number of days until anthesis (Reed, 2005) [20]. It was seen that all the three bud stages responded in varying degrees to callus induction on Gamborg B_5 medium endowed with BAP 2 mg/l and NAA 0.5 mg/l, but S_1 stage showed significantly a higher response towards callus induction rate than the other two stages. San Noeum and Gelebart (1986) [23] have highlighted the importance of developmental stage of embryo sac as well as flower buds to have a profound influence on *in vitro* gynogenesis. The present outcomes corroborate the results obtained by San Noeum and Gelebart (1986) [23] in barley, Sitbon (1981) [24] in gerbera, Ferrant and Bouharmont (1994) [6] in beetroot and Doi *et al*. (2010) [5] in gentians. Hence, bud stage may be an important determining factor in ovary culture of China aster.

One of the most important aspect of organogenesis in any crop is successful shoot regeneration. Cytokinins have been known to play a critical role for inducing shoot proliferation and in combination, auxins and cytokinins had shown effective shoot regeneration (Kumar *et al*., 2004) [16]. The BAP concentration has played a critical role in governing the shoot regeneration capacity of ovary derived calli of China Aster in our study. Our findings showed that MS medium supplemented with BAP 0.1 mg/l + NAA 0.01 mg/l had

performed excellently in comparison to other treatments with a shoot differentiation rate of 73.77 per cent. Our outcomes were in tantamount with the findings of Qi *et al.* (2011) [19] wherein MS medium with a combination of BAP 0.25 mg/l and NAA 0.3 mg/l was found to be the best differentiation medium with 70.5 per cent shoot differentiation rate during the anther culture of *Tagetes patula*. The BAP and NAA concentration used in shoot regeneration of anther derived calli of calla lily on MS medium, as reported by Zhang *et al.* (2011) [29] was found to be 1 mg/l and 0.1 mg/l respectively which were 10 times higher than our reported concentrations. In contrary to this, the experimental results were found to be superior to the observations of Miler and Muszczyk (2015) [17] who obtained shoot regeneration of just 10 per cent when ovary derived calli were cultured onto MS medium having BAP 1mg/l and 2,4-D 1 mg/l in chrysanthemum.

Table 1: Effect of different media on days for callus initiation and frequency of response of anthers and ovaries towards callus formation for China aster cv. AAC-1

Sl. No.	Medium	Growth Regulators Used	No. of anthers cultured	Frequency of anthers producing calli (%)	No. of days taken for callus initiation (Anthers)	No. of ovaries cultured	Frequency of ovaries producing Calli (%)	No. of days taken for callus initiation (Ovaries)
1	MS	BAP 2 mg/l + KN 2 mg/l + IAA 0.5 mg//l	60	0 (2.87)	0	60	25 (29.99)g	27.933e
2	C	BAP 2 mg/l + 2,4-D 0.1 mg/l	60	0 (2.87)	0	60	66.66 (54.7)d	29.067d
3	C_m	BAP 2 mg/l + 2,4-D 2 mg/l	60	0 (2.87)	0	60	50 (45)f	31.000bc
4	Nitsch and Nitsch	BAP 2 mg/l + NAA 0.5 mg/l	60	0 (2.87)	0	60	75 (60.012)c	31.933ab
5	N_6	BAP 2 mg/l + NAA 0.5 mg/l	60	0 (2.87)	0	60	58.3 (49.779)e	27.533e
6	NLN	BAP 2 mg/l + NAA 0.5 mg/l	60	0 (2.87)	0	60	83.3 (65.83)b	32.600a
7	B_5	BAP 2 mg/l + NAA 0.5 mg/l	60	33.61 (35.38)	28.69	60	93.3 (75.184)a	20.267f
8	Modified Ahmin and Vieth	BAP 0.2 mg/l + NAA 0.1 mg/l + IAA 0.1 mg//l	60	0 (2.87)	0	60	76 (51.71)e	30.800c
S.E.m±				0.079	0.65	-	0.851	0.331
CD (1%)				0.242	1.152	-	3.551	1.367

Note: The values in parenthesis indicate arc sin values

Table 2: Effect of different media on callus size and callus growth pattern for China aster cv. AAC-1

Sl. No.	Medium	Growth regulators used (mg/l)	Callus size from anthers (cm)	Callus growth pattern (Anthers)	Callus size from ovaries (cm)	Callus growth pattern(Ovaries)
1	MS	BAP 2 mg/l + KN 2 mg/l + IAA 0.5 mg//l	0	-	0.710 (1.099)b	Green/Granular
2	C	BAP 2 mg/l + 2,4-D 0.1 mg/l	0	-	0.608 (1.052)b	Green/Granular
3	C_m	BAP 2 mg/l + 2,4-D 2 mg/l	0	-	0.343 (0.917)c	Brown/Granular
4	Nitsch and Nitsch	BAP 2 mg/l + NAA 0.5 mg/l	0	-	0.288 (0.887)c	Green/Granular
5	N_6	BAP 2 mg/l + NAA 0.5 mg/l	0	-	0.295 (0.891)c	Greenish white/Granular
6	NLN	BAP 2 mg/l + NAA 0.5 mg/l	0	-	0.285 (0.886)c	Green/Granular
7	B_5	BAP 2 mg/l + NAA 0.5 mg/l	0.15	Brownish white	0.988 (1.219)a	Greenish white/Granular
8	Modified Ahmin and Vieth	BAP 0.2 mg/l + NAA 0.1 mg/l + IAA 0.1 mg//l	0	-	0.275 (0.880)c	Brown/Granular
S.E.m±			0.076	-	0.016	-
CD (1%)			0.252	-	0.075	-

Note: The values in the parenthesis indicate square root transformation values

Table 3: Effect of bud stages on ovary response towards callus formation, callus size and callus growth pattern cultured on Gamborg B_5 medium with BAP 2 mg/l + NAA 0.5 mg/l for China aster cv. AAC-1

Sl. No.	Bud stage	No. of ovaries cultured	Frequency of ovaries producing calli (%)	Callus size (cm)	Callus growth pattern
1	S_1	100	85.62 (67.72) a	0.981 1.407) a	Green/Granular
2	S_2	100	77.25 (61.51) b	0.855 (1.361) b	Greenish white/Granular
3	S_3	100	54.37 (47.51) c	0.636 (1.278) c	Greenish white/Granular
S.E.m±			0.26	0.015	
CD (1%)			3.04	0.087	

Note: The values in the parenthesis indicate arc sin values for frequency of ovaries producing calli (%) and square root transformation values for callus size

Table 4: Effect of different growth regulators on shoot differentiation rate and shoot length of ovary derived callus cultured on MS medium for China aster cv. AAC-1

Sl. No.	Growth regulators used (mg/l)	Total calli used for regeneration	Shoot differentiation rate (%)	Shoot length (cm)
1	-	24	20.97 (27.25)e	0.403 (0.950)d
2	BAP 0.1 mg/l + NAA 0.01 mg/l	24	73.77 (59.19)a	0.975 (1.214)a
3	BAP 0.2mg/l + NAA 0.01 mg/l	24	37.13 (37.54)c	0.785 (1.134)b
4	BAP 0.5 mg/l + NAA 0.01 mg/l l	24	25.20 (30.13)d	0.568 (1.033)c
5	BAP 1 mg/l + NAA 0.01 mg/l	24	46.39 (42.93)b	0.900 (1.183)a
S.E.m±			0.201	0.006
CD (1%)			1.619	0.051

Note: The values in the parenthesis indicate arc sin values for shoot differentiation rate and square root transformation values for shoot length.

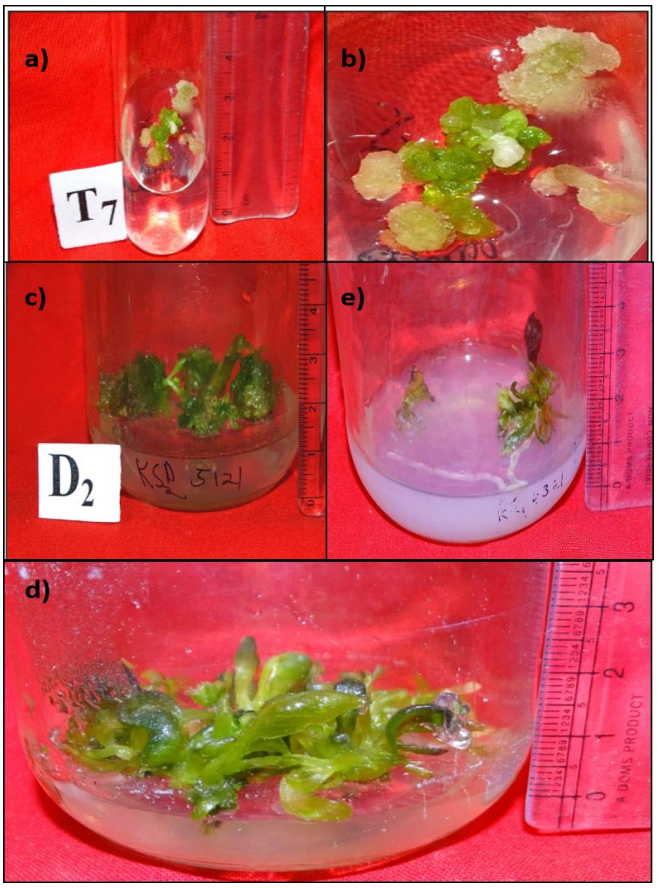

Fig 3a) Callus formation from ovaries of China aster cv. AAC-1 on B_5 medium with BAP 2 mg/l and NAA 0.5 mg/l; **b)** Enlarged view of calli; **c)** *in vitro* shoot initiation on MS medium with BAP 0.1 mg/l and NAA 0.01 mg/l; **d)** Shoot elongation MS medium with BAP 2 mg/l; e) *in vitro* rooting on half strength MS medium with IBA 1 mg/l

Conclusion

To the best of our knowledge, this is the first tissue culture study in China aster which could be considered as an initial step towards developing haploids in this crop. The callus produced on the best performing medium were granular and greenish white in colour. B_5 medium also performed exceptionally when compared to MS medium. Ammonium nitrate is a component of MS medium which has been recently banned due to its explosive nature and is not a component of B_5 medium which is also an added advantage.

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