Plant Cell Tiss Organ Cult (2016) 127:161–173
DOI 10.1007/s11240-016-1039-9

ORIGINAL ARTICLE

High-frequency in vitro flowering, hand-pollination and fruit setting in ten different cultivars of *Capsicum* spp. (*C. annuum*, *C. chinense*, and *C. frutescens*): an initial step towards in vitro hybrid production

Sk Moquammel Haque[1] · **Subhabrata Paul**[1] · **Biswajit Ghosh**[1]

Received: 30 November 2015 / Accepted: 6 July 2016 / Published online: 12 July 2016

Abstract We developed an efficient method for inducing high-frequency in vitro flowering and fruiting in 10 cultivars (Cap-1 to -10) of three different species of *Capsicum* (*C. annuum, C. chinense,* and *C. frutescens*) collected from diverse geographical regions of India and Mexico. Shoot tips of in vitro germinated seedlings were cultured on MS media supplemented with different concentrations of 6-benzylaminopurine (BAP) alone or in combination with silver nitrate or silver thiosulphate. Low BAP concentration was a good inducer of flower buds in vitro. Synergism between silver ions and cytokinin improved flower induction. Flowering frequency improved significantly when silver nitrate or silver thiosulphate was supplemented with optimum BAP. Maximum responses, 7.5 ± 0.20 (in Cap-1) to 15.8 ± 0.27 (in Cap-5) flowers, were induced in the medium containing 0.75 mg l^{-1} BAP and 30 μM silver thiosulphate within 60 days of culture. Both in vitro hand-pollination and high sucrose concentration (5.0–6.0 %) in the medium improved fruit setting. In general, fruits were very small (mini-fruit) and seedless; however, three cultivars (Cap-3, -5, and -6) produced normal fruits with seeds. On an average, 144 days were required from seed implantation to fruit ripening. The frequency of meiotic abnormalities was higher in flowers produced in vitro than in those produced in vivo, and pollen viability was relatively lower in the in vitro flowers than in the in vivo flowers. The present protocol offers a repeatable system for studying the physiological mechanism of flowering and fruiting as well as providing the basis for further investigation for rapid in vitro cross-breeding programmes in *Capsicum* spp. for commercial hybrid production.

Keywords Chilli pepper · Cytokinin · In vitro flowering · In vitro fruiting · Meiosis · Silver thiosulphate

✉ Biswajit Ghosh
ghosh_b2000@yahoo.co.in

Sk Moquammel Haque
sm.haque@yahoo.in

[1] Plant Biotechnology Laboratory, Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata 700118, India

Introduction

Capsicum (common name: chilli or peppers) is a commercially important crop of the Solanaceae family, which is cultivated worldwide for vegetables, spices, as a colouring agent, and for medicinal purposes. It includes 38 species, of which only six are cultivated, the major ones being *C. annuum*, *C. chinense,* and *C. frutescens* (Ramchiary et al. 2014). The Indian germplasm is mainly represented by *C. annuum* and *C. frutescens* with numerous cultivars (Thul et al. 2009); however a few cultivars of *C. chinense* are also cultivated in North-East India (Kehie et al. 2012). The fruits of *C. chinense* and *C. frutescens* are highly pungent and commonly used as a source of pungency in food, whereas those of *C. annuum*, usually known as sweet peppers, are pickled or used in curries. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is an alkaloid present in the fruits of *Capsicum*, which imparts the chilli its characteristic hot taste (Das et al. 2015; Kundu et al. 2015a). Numerous genetically diverse *Capsicum* cultivars are grown in different agro-climatic areas of India; particularly, West Bengal has the richest diversity in chilli (Paul et al. 2013). Since 2009, our laboratory is involved in specimen collection from different parts of India and abroad, ex situ conservation, and various other aspects such

as extraction and quantification of capsaicin, modulation of pungency, and investigating antioxidant property, and major bioactive compounds of the different cultivars of *Capsicum* (Paul et al. 2013; Das et al. 2015; Kundu et al. 2015a, b).

The life cycle of a flowering plant begins with seed germination followed by vegetative growth, flowering, and fruiting, and finally terminates with seeds development. Floral transition is one of the most significant events in the life cycle of flowering plants, during which the vegetative shoot apical meristem changes to a floral meristem (Kaur et al. 2015). *Capsicum* is a self-pollinated species; however, natural cross pollination results in the formation of variants within species or even within cultivars (Kehie et al. 2012). Plant breeders seek to generate interspecific crosses between *Capsicum* spp. for producing elite hybrids (Kamvorn et al. 2014; Manzur et al. 2015). Flowering in *Capsicum* is season-specific; therefore, the conventional crossing method is not feasible between genotypes flowering in different seasons or plants grown in different countries because of their extreme asynchrony of flowering. Under these circumstances, biotechnological approaches may overcome these barriers. In vitro flowering and fruiting has a great potential in breeding programmes for crop improvement (Sivanesan and Park 2015) and could be applied for rapid breeding of distant varieties through synchronisation of flowering (Franklin et al. 2000; Zhang 2007). It is advantageous over conventional breeding in terms of seasonal independency, completion of more than one breeding cycle within the same year, and reduced labour costs and space. In vitro techniques also facilitate the understanding of the physiology of flowering and fruiting through reduction of the influence of environmental factors and allow precise control of factors including plant growth regulators (PGRs), sugars, minerals, photoperiod, and temperature. To date, only a few reports are available on the induction of in vitro flowering in *C. annuum*. (Bodhipadma and Leung 2003) and *C. frutescens* (Tisserat and Galletta 1995; Sharma et al. 2008). However, all these reports dealt with a single cultivar of *Capsicum* and unlike other Solanaceous species, *Capsicum* is a recalcitrant genus in terms of in vitro culture (Kothari et al. 2010; Máthé et al. 2015). Therefore, the present study investigated 10 cultivars belonging to three different species of *Capsicum* (*C. annuum*, *C. chinense*, and *C. frutescens*), which possibly will help us understand the regulation of this recalcitrant genus. The inductive role of cytokinin in in vitro flowering has been investigated in several plant species (Taylor et al. 2005; Murthy et al. 2012), but never in *Capsicum*. Therefore, we investigated the role of cytokinin in in vitro flowering in *Capsicum* spp. To the best of our knowledge, this is the first comprehensive study on the synergistic effect of cytokinin and silver (Ag^+) ions on in vitro flowering of any plant species. The high-frequency in vitro flowering system described in this study may facilitate rapid breeding of *Capsicum*; it additionally offers a repeatable system for studying the physiological mechanism of flowering and fruiting.

Materials and methods

Plant materials, culture media, and culture conditions

Media preparation and culture conditions

Half-strength MS (Murashige and Skoog 1962) medium supplemented with 1.5 % (w/v) sucrose and 0.75 % (w/v) agar–agar was used for seed germination. Full strength MS medium supplemented with 0.75 % (w/v) agar–agar, 3.0 % sucrose, and different concentrations and combinations of 6-benzylaminopurine (BAP), silver nitrate ($AgNO_3$), and silver thiosulfate ($Ag_2O_3S_2$) were added according to the experimental requirement for in vitro flowering. Silver thiosulfate (STS) was added to the culture medium as a solution (20 mM), which was prepared by slowly pouring 20 ml of 100 mM silver nitrate (SN) solution into 80 ml of 100 mM sodium thiosulfate solution; and stored at 4 °C for further use up to 15 days. For in vitro fruit setting, different concentrations of sucrose were tested along with the respective medium used for flower induction only without any Ag^+ ions. pH of media was adjusted to 5.6 before adding agar–agar, and the media were autoclaved at 1.04 kg cm^{-2} pressure and 121 °C for 18 min. All cultures were incubated at 22 ± 2 °C under a 16-h light and 8-h dark cycle with a light intensity of 50 µmol m^{-2} s^{-1} using cool-white fluorescent lamps (Philips India Limited) in an environment-controlled chamber.

Surface sterilisation and in vitro seed germination

Detail of all the 10 cultivars of *Capsicum* spp. (Cap-1 to -10) used in the current study including their scientific name, local name/cultivar, area of collection, and geographical locations are presented in Table 1. Fresh and ripened red chillies of the seven Indian cultivars were collected from markets of different regions of India. Few seeds were sown inside the poly-house of our college (RKMVC College, Rahara, Kolkata, India) for studying in vivo plants and the remaining seeds were used for in vitro culture. Seeds of the Mexican cultivars were collected from the Chile Pepper Institute, New Mexico State University, Mexico. These seeds were surface-sterilised sequentially in 2.5 % (w/v) systemic fungicide

Table 1 Detail of the ten cultivars of *Capsicum* spp. used in this experiment

Code name	Scientific name	Local name (cultivar)	Collection area/source	
			District, state, countries	Geographic location (latitude and longitude)
Cap-1	*Capsicum chinense*	Bhut Jolokia	Dimapur, Nagaland, India	25°48′N, 93°47′E
Cap-2	*Capsicum frutescens*	Dhani Lanka	Nadia, West Bengal, India	23°24′N, 88°30′E
Cap-3	*Capsicum frutescens*	Kull Lanka	Murshidabad, West Bengal, India	24°08′N, 88°16′E
Cap-4	*Capsicum annuum*	Kalo Lanka	Cooch Behar, West Bengal, India	26°22′N, 89°29′E
Cap-5	*Capsicum annuum*	Jhanti Lanka	Howrah, West Bengal, India	22°36′N, 87°92′E
Cap-6	*Capsicum annuum*	Bullet Lanka	Malda, West Bengal, India	25°00′N, 88°09′E
Cap-7	*Capsicum annuum*	Acchar Lanka	Shamshabad, Hyderabad, India	17°15′N, 78°23′E
Cap-8	*Capsicum annuum*	deArbol	The Chile Paper Institute, Mexico	32°16′N 106°44′W
Cap-9	*Capsicum annuum*	NuMex Mirasol	The Chile Paper Institute, Mexico	32°16′N 106°44′W
Cap-10	*Capsicum annuum*	Serrano	The Chile Paper Institute, Mexico	32°16′N 106°44′W

(Bavistin®) for 12 min, 5.0 % (v/v) liquid detergent (Tween-20) for 5 min, and 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 10 min, and finally rinsed three times (5 min each) in sterile distilled water to remove traces of $HgCl_2$. Next, the surface-sterilised seeds were aseptically inoculated on half-strength MS basal medium and kept in a dark chamber for germination.

Induction of in vitro flowering

For in vitro flower induction, shoot tips (approximately 1.5–2.5 cm) of the in vitro grown seedlings were cultured on MS media supplemented with different concentrations of BAP alone or in combination with SN or STS and subcultured at 60-day intervals (Table 2). For the maturation of the flower bud up to the blooming stage, the effect of the aeration system of different types of culture vessels [25 × 150 mm and 32 × 200 mm culture tubes, 250 ml conical flask (Borosil®), plugged with 'cotton-plug', and culture bottle (sealed with a plastic cap)] were tested.

In vitro pollination and fruit setting

For in vitro pollination, flowers were hand-pollinated in a laminar airflow hood using sterile forceps and needles. Freshly bloomed flowers were pollinated with the anthers of the flowers bloomed 3–4 days earlier. Self-pollination (within the same plant), cross-pollination (between different plants of the same species), and reciprocal pollination (between two different species) were performed artificially. After pollination, the plantlets were observed for fruit formation. The effect of different concentrations (2, 3, 4, 5, and 6 %) of sucrose on fruit setting was tested.

Meiotic study of in vivo and in vitro flower buds

Flower buds of appropriate sizes (2.0–3.5 mm) were collected from both in vivo and in vitro plants and fixed in absolute ethanol and glacial acetic acid (3:1 v/v) for 24 h at 4 °C. After overnight fixation, the buds were either stored in 70 % ethanol at 4 °C or worked with immediately. Anthers were smeared in 2.0 % acetocarmine following Haque and Ghosh (2016a). Nine flower buds from randomly selected three plants of each cultivar and a minimum of 100 pollen mother cells (PMCs) from each flower bud were observed. Different meiotic stages were observed under a Leica DM750 microscope at 40× magnification and photographed using a Leica DFC295 camera.

Pollen viability of in vivo and in vitro flowers

Pollen viability of both in vivo and in vitro plants was separately measured by staining with 2.0 % acetocarmine following Haque and Ghosh (2013c). Nine flowers from three randomly selected plants of each cultivar and a minimum of 300 pollen grains from each flower were observed. The percentage of viability was determined by dividing the number of viable pollens by the total number of pollens per field of view and multiplying by 100.

Statistical analysis

In the present experimental design, each treatment of flowering and fruiting was repeated three times with 10 explants per treatment. Flowering and fruiting were observed and recorded at every 3-day interval. All data were subjected to one-way analysis of variance (ANOVA) using the SPSS software for Windows (IBM® SPSS, version 21.0, Chicago,

Table 2 Effect of cytokinin (BAP) and silver ions (SN and STS) on in vitro flower induction in ten different cultivars of *Capsicum* spp

BAP (mg l^{-1})	SN (μM)	STS (μM)	Total number of bloomed flower per plant up to 60 days of implantation									
			Cap-1	Cap-2	Cap-3	Cap-4	Cap-5	Cap-6	Cap-7	Cap-8	Cap-9	Cap-10
0	–	–	0^{a}	0.5 ± 0.12^{a}	0^{a}	0.6 ± 0.06^{a}	0.8 ± 0.10^{a}	0^{a}	0^{a}	0.4 ± 0.02^{a}	0^{a}	0^{a}
0.25	–	–	0.7 ± 0.13^{ab}	2.7 ± 0.11^{bc}	0.7 ± 0.08^{ab}	1.8 ± 0.09^{b}	2.1 ± 0.12^{b}	1.4 ± 0.11^{b}	0^{a}	1.6 ± 0.05^{ab}	1.1 ± 0.03^{ab}	0.4 ± 0.03^{ab}
0.50	–	–	1.7 ± 0.14^{c}	3.5 ± 0.14^{c}	1.9 ± 0.14^{b}	4.0 ± 0.13^{c}	4.5 ± 0.18^{c}	3.3 ± 0.08^{c}	2.1 ± 0.03^{b}	3.8 ± 0.08^{b}	3.4 ± 0.09^{c}	2.3 ± 0.09^{bc}
0.75	–	–	3.2 ± 0.18^{d}	4.8 ± 0.12^{d}	4.2 ± 0.12^{cd}	6.3 ± 0.11^{d}	7.2 ± 0.22^{d}	5.1 ± 0.12^{cd}	4.3 ± 08^{c}	5.1 ± 0.11^{c}	4.1 ± 0.11^{cd}	4.0 ± 0.11^{d}
1.00	–	–	2.8 ± 0.15^{d}	4.1 ± 0.20^{cd}	3.6 ± 0.15^{c}	5.1 ± 0.14^{cd}	6.9 ± 0.25^{d}	5.6 ± 14^{d}	4.5 ± 12^{c}	3.4 ± 0.07^{b}	1.9 ± 0.05^{b}	3.6 ± 0.07^{cd}
1.50	–	–	1.3 ± 0.09^{bc}	2.2 ± 0.17^{b}	1.6 ± 0.13^{b}	2.7 ± 0.05^{bc}	2.9 ± 0.16^{b}	3.5 ± 11^{c}	2.4 ± 06^{b}	1.2 ± 0.03^{ab}	0.8 ± 0.02^{ab}	2.8 ± 0.06^{c}
0.75	15	–	4.7 ± 0.18^{e}	7.9 ± 0.14^{e}	4.9 ± 0.15^{d}	7.7 ± 0.12^{e}	9.4 ± 0.28^{e}	7.8 ± 19^{e}	6.1 ± 11^{d}	6.8 ± 0.12^{cd}	4.7 ± 0.12^{cd}	6.2 ± 0.12^{e}
0.75	30	–	5.8 ± 0.18^{f}	8.3 ± 0.18^{e}	5.8 ± 0.11^{de}	9.1 ± 0.20^{f}	10.3 ± 0.31^{ef}	9.6 ± 17^{g}	7.5 ± 14^{e}	7.5 ± 0.10^{d}	6.0 ± 0.13^{de}	7.4 ± 0.11^{ef}
0.75	45	–	6.2 ± 0.19^{f}	9.4 ± 0.09^{f}	5.5 ± 0.09^{de}	8.3 ± 0.18^{ef}	12.4 ± 0.27^{g}	9.0 ± 21^{fg}	7.2 ± 12^{de}	8.0 ± 0.15^{de}	5.4 ± 0.15^{d}	8.1 ± 0.14^{f}
0.75	–	15	5.8 ± 0.19^{f}	8.1 ± 0.23^{e}	7.2 ± 0.14^{f}	9.2 ± 0.17^{f}	11.2 ± 0.31^{f}	8.1 ± 18^{ef}	8.4 ± 15^{ef}	8.6 ± 0.17^{ef}	7.1 ± 0.16^{e}	8.7 ± 0.12^{fg}
0.75	–	30	7.5 ± 0.20^{g}	8.8 ± 0.19^{ef}	8.1 ± 0.16^{g}	11.5 ± 0.15^{g}	15.8 ± 0.27^{i}	8.4 ± 15^{f}	10.4 ± 0.21^{g}	9.2 ± 0.16^{f}	8.3 ± 0.14^{ef}	11.8 ± 0.26^{i}
0.75	–	45	7.2 ± 0.18^{g}	9.2 ± 0.22^{f}	7.7 ± 0.13^{fg}	12.3 ± 0.21^{gh}	14.1 ± 0.23^{h}	7.5 ± 13^{de}	9.7 ± 19^{fg}	8.4 ± 0.14^{e}	8.9 ± 0.18^{f}	10.3 ± 0.21^{h}

Each value represents the mean ± standard error, n = 30. Mean followed by the same letters in each column are not significantly different at $P < 0.05$ according to Tukey's multiple range tests

BAP 6-benzylaminopurine, *SN* silver nitrate, *STS* silver thiosulphate

IL). After conducting ANOVA, the means were further separated using Tukey's test at $P \leq 0.05$.

Results

The seeds of all 10 cultivars were germinated aseptically. However, the days required for germination (5–14 days) as well as the percentage of germination (42.2–93.3 %) varied for different cultivars. After germination, the seedlings were transferred to MS basal medium without any PGR and grown up to 4.5–5.0 cm with 3–4 nodes.

Effect of PGR (BAP) on flower bud induction

Depending on the cultivars, the first flower bud was induced within 17–31 days of culture. Three cultivars—Cap-4, -5, and -10—exhibited early flower bud induction (within 17–23 days) and produced higher number of flowers than the other cultivars—Cap-1, -2, -3, -6, -7, -8, and -9—that took longer time (24–31 days) for flower bud induction. In the control (PGR-free) medium, very few flower buds were induced in only four (Cap-2, -4, -5, and -8) of the 10 cultivars. However, low BAP concentration stimulated in vitro flower bud induction. Depending on the cultivars, an optimum response was observed with 0.75–1.0 mg l^{-1} BAP (Table 2). Of the 10 cultivars, the highest number (7.2 ± 0.22) of flower buds were induced in Cap-5 on MS medium containing 0.75 mg l^{-1} BAP alone. Higher BAP concentration (above the optimum) had a negative effect and the number of flower buds decreased in all the 10 cultivars (Table 2). Flower buds were initially induced in shoots without roots (Fig. 1a–c, e, i–l); however, gradually these shoots developed roots and flowered continuously (Fig. 1d, f, g).

Effect of SN and STS on flower bud induction

Both SN and STS at low concentrations in combination with optimum BAP enhanced the rate of in vitro flower bud induction; however, STS was more effective than SN. Depending on the cultivars, the best response was observed in the medium containing 30–45 μM STS and an optimum BAP concentration (Table 2). Maximum 7.5 ± 0.20 (Cap-1) to 15.8 ± 0.27 (Cap-5) number of flowers were induced within 60 days of culture on MS medium containing 0.75 mg l^{-1} BAP and 30 μM STS.

Effect of aeration system of the culture vessel on maturation of flower buds

The aeration system of the culture vessel significantly affected the growth and development of the flower buds to

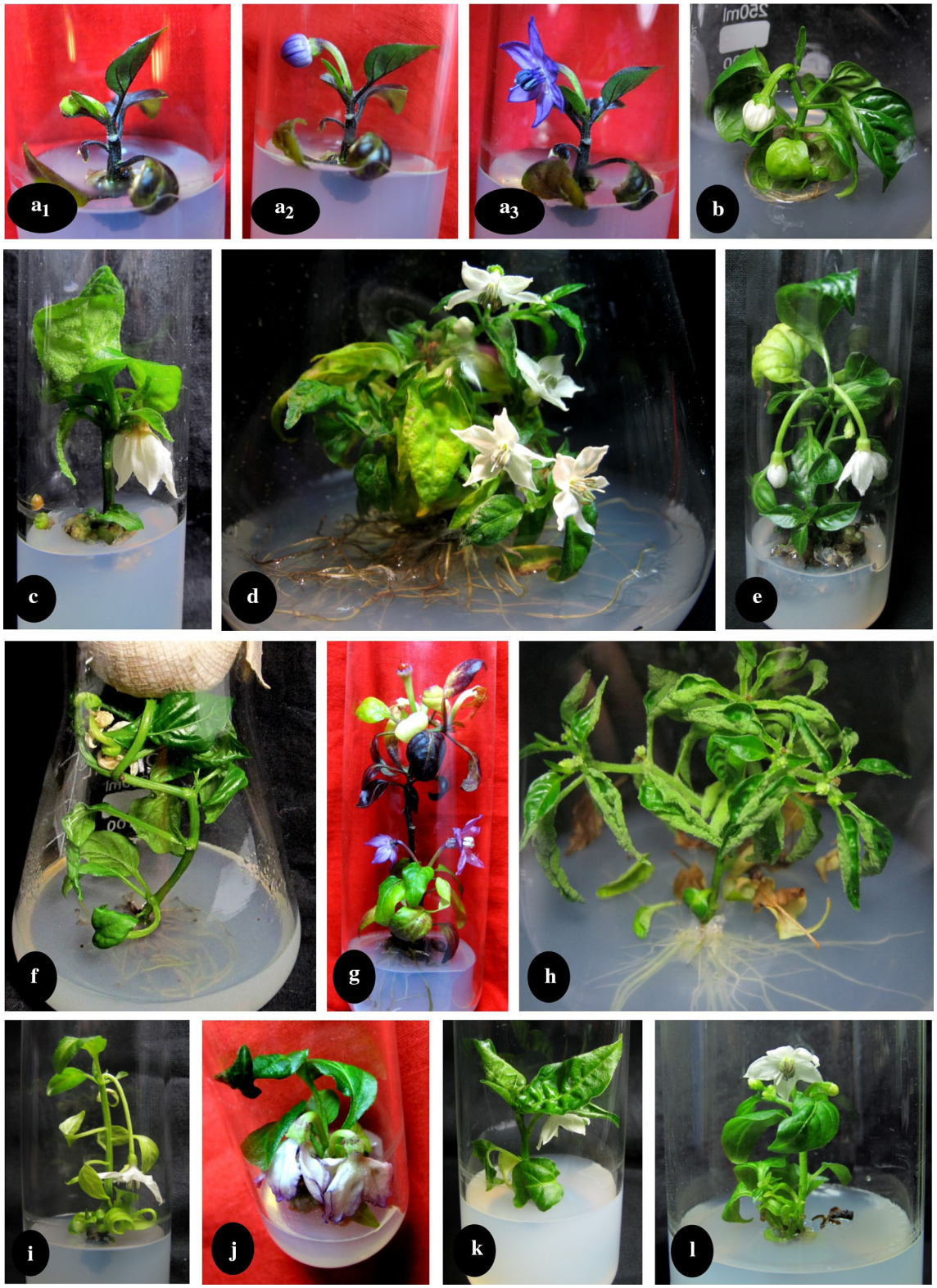

Fig. 1 In vitro flowering in different cultivars of *Capsicum*. **a**$_1$, **a**$_2$, and **a**$_3$ Successive development of the Cap-4 flower bud up to blooming. **b** Flower bud of Cap-6. **c** Flower of Cap-10. **d** Flowers of Cap-5. **e** Flowers of Cap-9. **f** Flower of Cap-3. **g** Flowers of Cap-4. **h** Immature flower buds of Cap-5 cultured on air-tight jam bottle, all buds dropped before blooming. **i** Flower of Cap-1. **j** Flowers of Cap-7. **k** Flower of Cap-2. **l** Flower of Cap-8. (Color figure online)

attain the blooming stage. When the plants were cultured in airtight culture bottles capped with plastic caps, whitish callus-like structures were observed on the dorsal surface of the leaves as well as on the entire surfaces of the young flower buds (Fig. 1h). Most of the buds dropped during their young stage; however, this problem was overcome and all the buds reached the blooming stage when the plants were cultured in well-aerated culture vessels (culture tubes or conical flasks plugged with cotton plugs). On an average, from the day of bud induction, 19–24 days were required for flower blooming (Fig. 1a_1–a_3), and these in vitro bloomed flowers sustained for 4–5 days. In general, flowers bloomed one-by-one in a continuous manner and maximum four flowers bloomed at a time in Cap-5 (Fig. 1d). These in vitro-bloomed flowers presented normal morphological characteristics; they were normal in size with white, whitish-violet, or violet petals (Fig. 1a–g, i–l), which were similar to the in vivo-bloomed flowers.

Effect of hand-pollination and sucrose concentration on fruit development

In vitro hand pollinations improved the percentage of fruit set and were successful in 8 of the 10 cultivars. Most of the in vitro-developed fruits were too small (3–5 mm) and seedless; these fruits were called 'mini-fruits' (Fig. 2a, b). High concentrations of sucrose (5–6 % w/v) led to an increase in the fruit-setting percentage (Fig. 3). Of the 10 cultivars, two (Cap-1 and -9) failed to develop fruits, five (Cap- 2, -4, -7, -8, and -10) produced mini-fruits only, and three (Cap-3, -5, and -6) produced both mini-fruits as well as comparatively large normal fruits with seeds (Fig. 2c–f). However, in the current study, these three cultivars (Cap-3, -5, and -6) even failed to produce normal fruits when pollinated with different species (i.e. reciprocal pollinations). Hence, normal fruits were produced through self- and/or cross-pollinations only. From the day of pollination, approximately 58–65 days were required for the fruits to reach the ripening stage. The deep-green mature fruits required 11–17 days to turn completely red (Fig. 2c_1–c_3). A maximum of 6.6 ± 0.32 fruits developed in Cap-3 on MS medium supplemented with 5.0 % (w/v) sucrose, of which 3.5 ± 0.36 were mini-fruits and the remaining 3.1 ± 0.28 were normal fruits (Fig. 3). Flowers were continuously induced even after fruit setting (Figs. 1g, 3b). On an average, the total time required from in vitro seed implantation to fruit ripening was 144 days (Fig. 4). The in vitro fruits were relatively smaller than the fruits of the poly-house grown plants and contained none or just a few seeds (Fig. 2f). Unfortunately, none of the in vitro-produced seeds germinated; nonetheless, this protocol serves as an initial step towards the in vitro production of hybrid seeds. Research to manipulate this protocol for viable seed production is ongoing.

Meiotic status of in vivo and in vitro flowers

Light microscopic investigations revealed that all the meiotic events of in vitro flowers were normal and similar to those of the in vivo flowers. Different normal stages along with few abnormalities were observed during meiosis (Fig. 5a–w). Perfect chromosome pairing with 12 bivalents was observed at the diakinesis stage of prophase-I (Fig. 5b). Cytokinesis was of the simultaneous type, that is, no cell plates appeared after the completion of meiosis-I. Various types of meiotic abnormalities were observed in both in vitro and in vivo plants. Comparative results of the total meiotic abnormalities of the in vitro and in vivo flowers are summarised in a graphical figure (Fig. 6a). However, overall results show that the abnormality percentage was slightly higher in the in vitro flowers than in the in vivo flowers. Occasionally, few bivalent or univalent chromosome(s) failed to attain the equatorial plane during metaphase-I (Fig. 5i–l). Other abnormalities such as 'univalent laggards', 'bivalent laggards', 'sticky bridge, 'late separation', 'early separation', 'dicentric bridge', and 'acentric fragment' were observed during meiosis-I (Fig. 5m–v) and meiosis-II (Fig. 5w).

Pollen viability of in vivo and in vitro flowers

Acetocarmine staining revealed a very good contrast between viable and non-viable pollen grains (Fig. 7a, b). The viability percentages varied widely among different cultivars (Fig. 6b). However, the overall results showed that pollen viability of in vitro flowers was significantly lower than that of in vivo flowers of the same cultivars (Fig. 6b).

Discussion

In vitro flowering

Capsicum is a recalcitrant plant in terms of in vitro cell, tissue, and organ culture (Kothari et al. 2010; Máthé et al. 2015). Because of its high genotypic dependence and recalcitrant nature, the in vitro growth of *Capsicum* is comparatively slower than that of other Solanaceous members (Ochoa-Alejo and Ramirez-Malagon 2001; Kehie et al. 2012). In vitro flowering is presumably the most elusive and most fascinating of all in vitro plant developmental processes (da Silva et al. 2014). The first aim of the present study was to establish a general protocol for high-frequency in vitro flowering of ten cultivars of *Capsicum*. In vitro flowering is crucial in selective hybridisation, particularly in using pollen from rare stocks, and may be the first step towards the possibility of recombining genetic

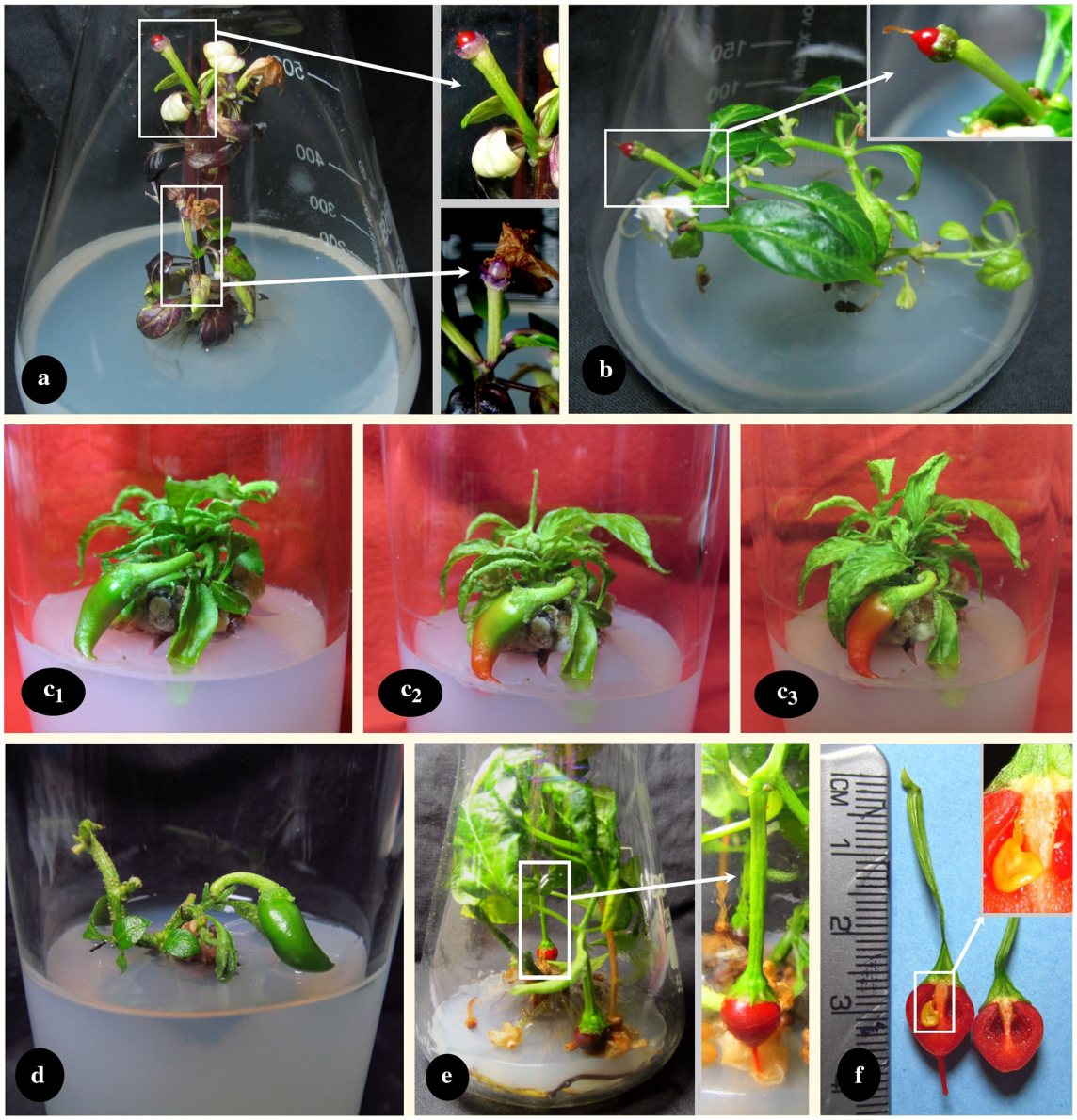

Fig. 2 In vitro fruiting in different cultivars of *Capsicum*. **a** In vitro plant of Cap-4 bearing young (*violet*) as well as ripen (*red*) mini-fruits. **b** Ripen mini-fruit bearing plant of Cap-10. $\mathbf{c_1}$, $\mathbf{c_2}$, and $\mathbf{c_3}$ Successive in vitro development of Cap-5 fruit up to ripening. **d** In vitro developed mature *green* fruit of Cap-6. **e** In vitro plant of Cap-3 bears two ripen fruits. **f** Longitudinal section of the in vitro developed fruit of Cap-3 containing seed. (Color figure online)

material via in vitro fertilisation in nonhybridisable lines (Murthy et al. 2012). Flowering is a complex process that is regulated by a combination of genetic and environmental factors, including PGRs, carbohydrates, light, and pH of the culture medium (Murthy et al. 2012). Flowering under in vitro conditions can decrease the influence of environmental factors and allow the precise control of factors, such as temperature, light, and pH, and application of PGRs, sucrose, STS, and SN to understand their individual role in flowering and fruiting.

Our experimental design completely differs from that followed in previous studies on in vitro flowering of *Capsicum*. Bodhipadma and Leung (2003) reported the enhancing role of auxin (α-naphthaleneacetic acid) in the in vitro flowering of *C. annuum*; however, they observed only 3.8 flowers per plant. The in vitro flowering protocol was further improved by Sharma et al. (2008) to achieve maximum 7.0 flowers per plant using SN in *C. frutescens*. Presently, we have successfully induced maximum 15.8 flowers per plant, which is much higher than those reported in the previous studies. In the current study, we examined the influence of BAP on flower induction in *Capsicum*; and BAP proved beneficial. When all other parameters were maintained at the optimal levels, the change in the BAP

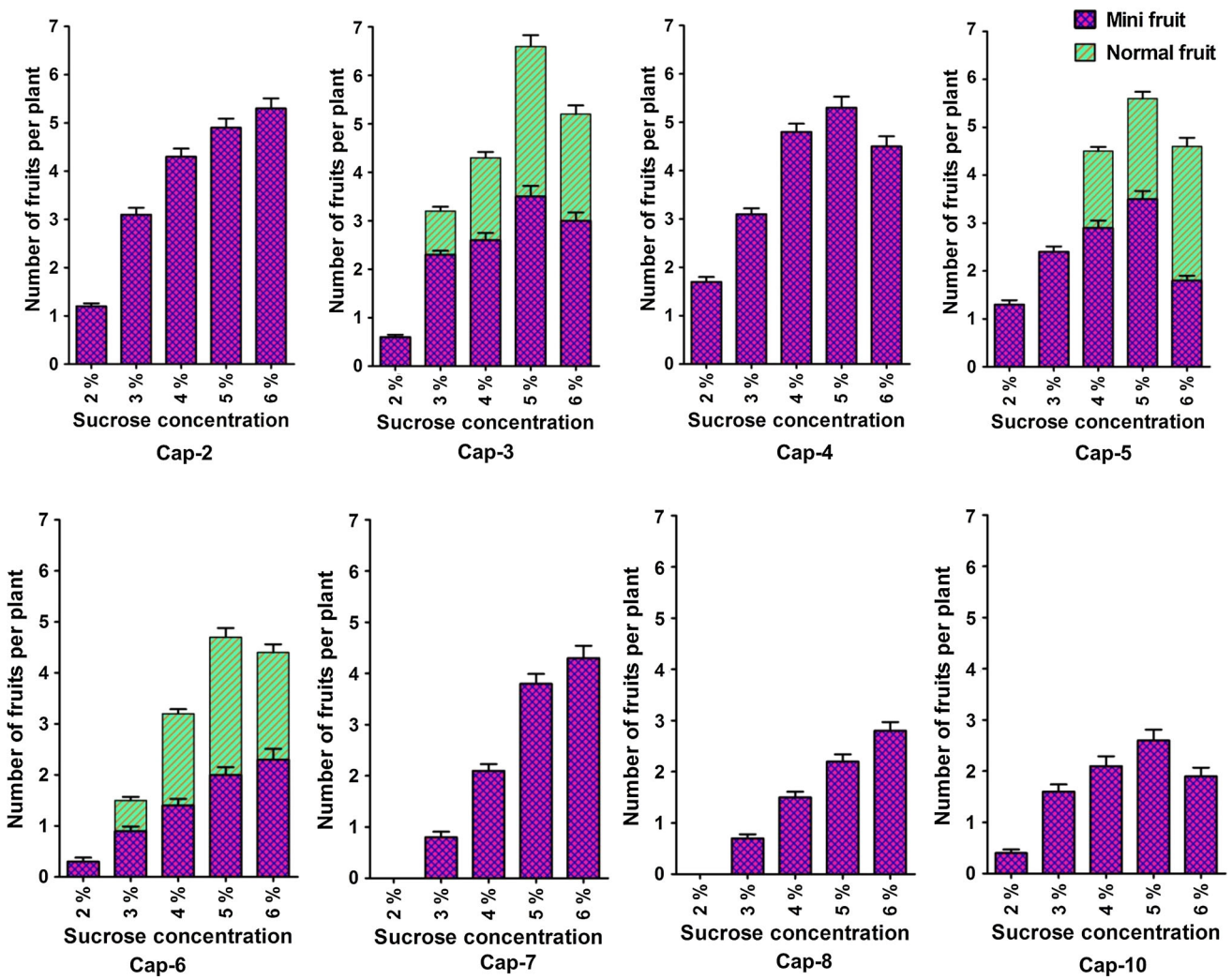

Fig. 3 Effect of different sucrose concentrations on in vitro fruiting of eight cultivars of *Capsicum*

concentration affected the in vitro flowering of *Capsicum*; concentrations higher or lower than the optimal concentration had a negative effect. Similarly, the role of BAP in in vitro flower inductions was previously reported in several plant studies, including those on *Momordica*, *Kniphofia, Perilla, Ipomoea,* and *Bacopa* (Wang et al. 2001; Taylor et al. 2005; Zhang 2007; Haque and Ghosh 2013b, c) and even another Solanaceous plant, *Withania* (Sivanesan and Park 2015).

In bamboo, flowers could be induced only in the absence of roots, when the shoots were cultured on a medium containing BAP (Joshi and Nadgauda 1997), whereas, in *Perilla*, shoots without roots did not produce flowers (Zhang 2007). However, present findings in *Capsicum* spp. revealed that the flower buds were induced in both shoots without roots and rooted shoots.

Ethylene, a potential stimulant of flower senescence (Beyer 1976), is synthesised by plants during certain stages of development and in response to abiotic and biotic stresses (Bakshi et al. 2015). Our results revealed that the leaves and young flower buds dropped when the plants were cultured in airtight containers; however, this problem was resolved when they were cultured in well-ventilated culture vessels plugged with cotton plugs. The ethylene level in the airtight culture vessels might inhibit the growth and development of the young flower buds of *Capsicum* and may stimulate their senescence. Similar findings were observed during studies on in vitro flowering and seed-set in *C. annuum* and different pea genotypes, where accumulated ethylene was considered to hinder the growth and development of immature floral buds (Bodhipadma and Leung 2003; Ribalta et al. 2014). The ventilation system may help in releasing the accumulated ethylene from the culture vessels and circuitously preventing bud abscission. On the other hand, Ag^{+} ions are well-known as effective, specific, non-competitive inhibitors of ethylene action

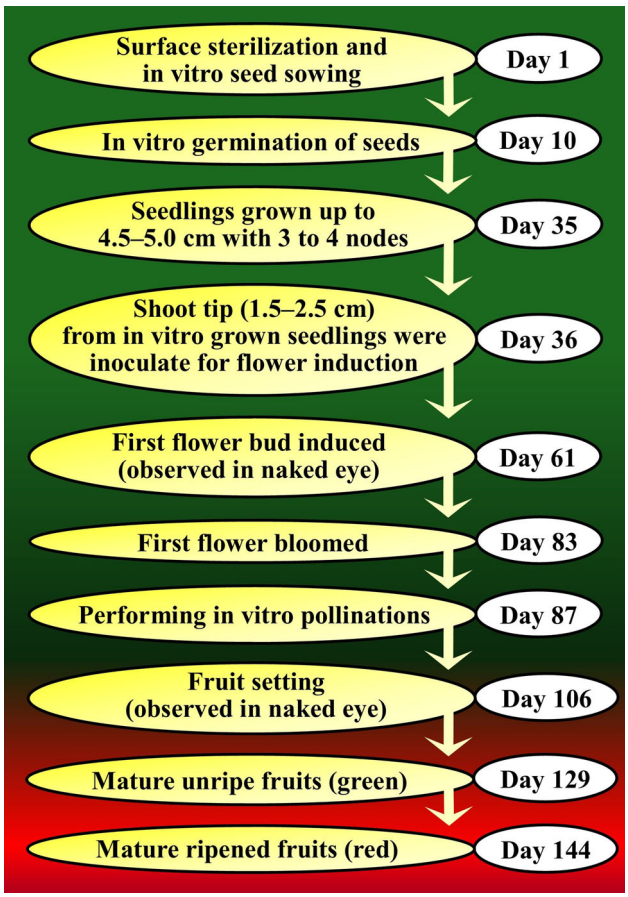

Fig. 4 An average timeline for the completion of overall process from in vitro seed sowing to fruit ripening in *Capsicum* spp.

(Beyer 1976) and are involved in flower induction responses (Bais et al. 2000; Sharma et al. 2008). The enhancing effect of STS and SN on in vitro flowering was previously reported in chicory and *Capsicum* (Bais et al. 2000; Bodhipadma and Leung 2003; Sharma et al. 2008). In plant tissue culture, Ag^+ ions have been widely used either as nitrate (i.e. SN) or as the more mobile thiosulphate (i.e. STS) (Würschum et al. 2015). According to our findings, STS is more effective than SN in enhancing in vitro flower bud induction and maturation. SN is not too mobile within the plant, whereas STS is a more translocatable form of the ethylene action inhibitor (Aloni et al. 1995; Hoyer 1998); therefore, STS may be more efficient than SN. The inhibitory effect of STS on flower and fruit abscission has been previously documented in glasshouse-grown *C. annuum* plants (Aloni et al. 1995; Hoyer 1998). Our findings revealed that the stimulating effect of BAP on flower induction was further improved by supplementing SN and STS in the culture medium. From the present findings, we can assume that in vitro flower bud induction and maturation was regulated by the synergistic effect of cytokinin and Ag^+ ions. The effect of cytokinin on in vitro flowering has been studied by many researchers (Wang et al. 2001; Taylor et al. 2005; Zhang 2007; Haque and Ghosh 2013b, c). Similarly, the effect of Ag^+ ions on in vitro flowering has been reported by few studies (Bais et al. 2000; Bodhipadma and Leung 2003; Sharma et al. 2008); however, their synergistic effects, which are beneficial for *Capsicum* and may be effective in different plant species, have never been studied.

In vitro fruiting

The second aim of the present study was to standardise a protocol for in vitro fruit setting for different cultivars of *Capsicum*. Although *Capsicum* is primarily a self-pollinating plant, the in vitro condition may inhibit self-pollination, which demands artificial hand-pollination for fertilisation and further development of seeds. In vitro fruiting and formation of viable seeds could occur without hand pollination in *Centaurium* (Todorović et al. 2006), *Lens* (Sarker et al. 2012), pea (Ribalta et al. 2014), *Scrophularia* (Jeong and Sivanesan 2015), and other Solanaceous members including *Lycopersicon* (Sheeja and Mandal 2003), *Withania* (Sivanandan et al. 2015; Sivanesan and Park 2015), and even *C. fruitescens* (Tisserat and Galletta 1995). In contrast, in vitro fruiting in *Ipomoea* (Haque and Ghosh 2013b) and *C. annuum* (Bodhipadma and Leung 2003) was possible only following hand pollination. The majority of the non-pollinated flowers of *Capsicum* dropped but did not develop into fruits. According to the previous reports, in vitro fruiting was observed only following hand pollination in *C. annuum* (Bodhipadma and Leung 2003) and without any hand pollination in *C. fruitescens* (Tisserat and Galletta 1995). Unfortunately, artificial reciprocal pollination did not result in seed-bearing normal fruits. A similar approach was adopted by Sim et al. (2007) in *Dendrobium* orchid; however, they also failed to develop fruits in this plant.

Ethylene plays a vital role in initiating and accelerating ripening-related processes (Kumar et al. 2009); therefore, we had withdrawn the ethylene inhibitor (i.e. Ag^+ ions) from the fruit development medium to accelerate fruit ripening.

In vitro breeding is required for overcoming a cross between distantly related species so as to include maximum natural genetic variation in the breeding lines (De Jeu 2000). The present study is the first attempt in in vitro reciprocal pollination between two different species of *Capsicum*, some of which developed into ripe mini-fruits; however, the resulting fruits were seedless. Hence, additional studies are required for the establishment of effective protocols for the production of viable seeds as a result of in vitro reciprocal pollination; if successful, this system

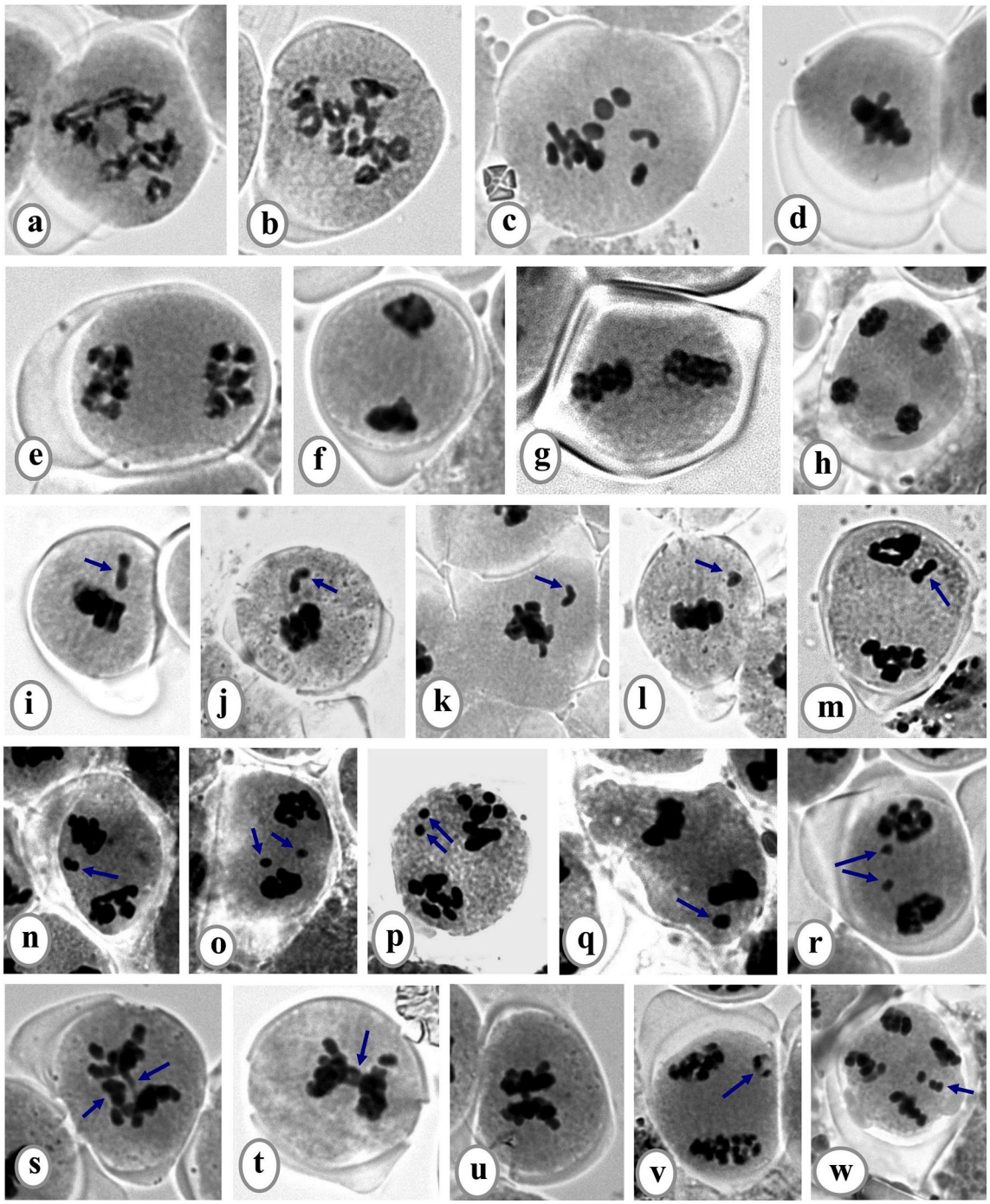

Fig. 5 Acetocarmine stained normal (**a**–**h**) and abnormal (**i**–**w**) meiotic stages of in vitro flower buds of different *Capsicum* spp. **a** Diplotene stage. **b** Diakinesis showing 12 bivalent chromosomes. **c**, **d** Metaphase- I. **e** Anaphase-I showing two set of chromosomes are moving towards the opposite poles. **f** Telophase-I showing two set of chromosomes are reached on two opposite poles. **g** Metaphase-II. **h** Telophase-II. **i**–**l** Metaphase-I with one bivalent don't reach on the equatorial plane. **m**, **n** Anaphase-I with one bivalent laggard. **o**, **p** Anaphase-I with two univalent laggards. **q** Anaphase-I with early separation. **r** Anaphase-I with late separation. **s** Telophase-I with dicentric bridge and acentric chromosome. **t**, **u** Telophase-I with sticky bridge. **v** Telophase-I with multi-polarity. **w** Anaphase-II with three laggard chromosomes

will be highly beneficial for the breeder. Because in vitro flowering occurs throughout the year, and on an average 144 days are required from seed sowing to fruit ripening (Fig. 4), at least two breeding cycles are completed within the same calendar year, which shortens the time required for conventional breeding (by at least 1 year), reduces the labour costs, and optimises the space required for conventional breeding.

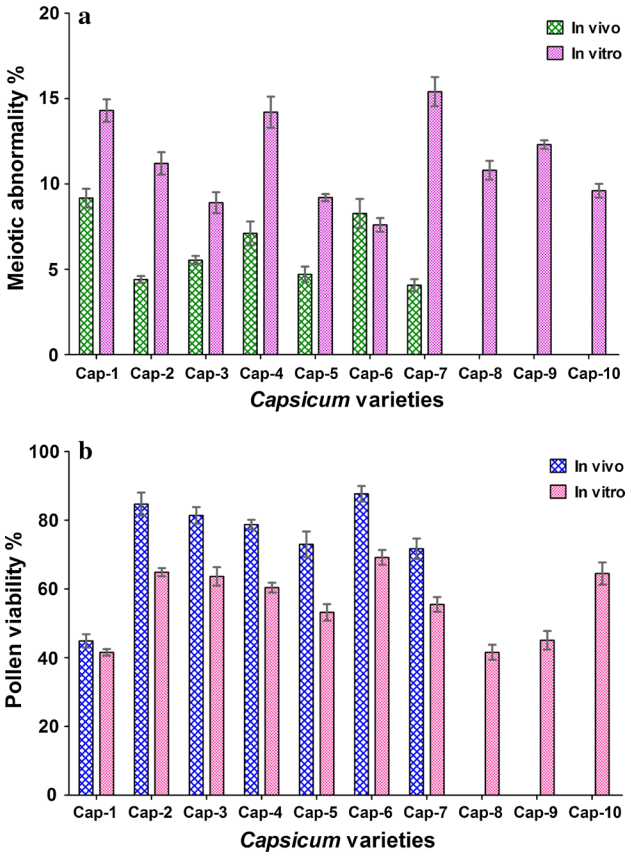

Fig. 6 Comparative results of in vivo and in vitro plants of ten cultivars of *Capsicum* spp. **a** Meiotic abnormalities. **b** Pollen viability

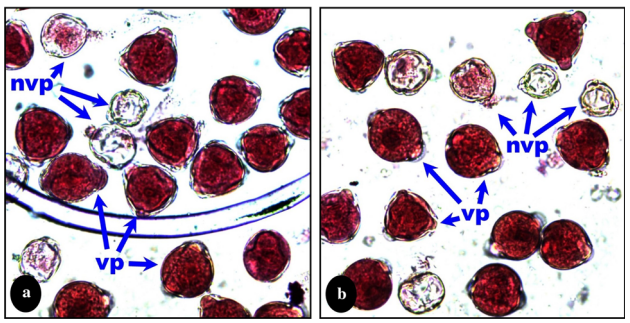

Fig. 7 Pollen viability of *Capsicum* by acetocarmine staining. **a** Pollen grains of in vitro flower. **b** Pollen grains of in vivo flower. (*vp* viable pollen, *nvp* non-viable pollen)

The growth and development of fruits require high energy, which results in a significant drop in nutrients in the culture medium. Therefore, supplying sufficient carbon sources is necessary for the proper in vitro growth and development of fruits. Sucrose is the most favoured carbon source for flower induction and fruit setting in Solanaceous members (Sivanandan et al. 2015). Our results showed that the fruit setting percentage of *Capsicum* was improved with increasing sucrose concentration (Fig. 3). Similar findings were also noted in *Centaurium, Ipomoea*, and *Withania* where higher concentrations of sucrose were required for the development of fruit and seeds (Todorović et al. 2006; Haque and Ghosh 2013b; Sivanandan et al. 2015). However, our experimental results revealed that with the same treatment, two cultivars failed to produce fruits, whereas five cultivars produced only mini-fruits, and rest of the three cultivars produced both mini-fruits as well as normal fruits with seeds. This may be due to the recalcitrant nature of this genus. We had previously successfully developed a protocol for in vitro completion of the sexual life cycle and production of next-generation (regenerants-1) plants of *Ipomoea quamoclit* (Haque and Ghosh 2013b); however, during the present study, we were unsuccessful in developing viable seeds of *Capsicum*. Further research to overcome this problem is in progress.

Meiotic status and pollen viability of in vivo and in vitro flowers

This is the first report describing the meiotic behaviour of in vitro flowers in not only *Capsicum* but also any plant species. Meiosis is the principal event of sexual reproduction, which controls the gene flow from one generation to the next. Changes in chromosome number and structure are the most frequently observed chromosomal abnormalities in tissue culture-regenerated plants (Tomiczak et al. 2015). These chromosomal changes also affect the meiotic behaviour, resulting in pollen sterility and ultimately affecting the fertility of the regenerated plants. Although the meiotic behaviour of in vitro flowers has not been studied in any plant until now, the meiotic study of the tissue culture-derived ex vitro plants has been reported in *Aloe, Drimiopsis* and *Ledebouria* (Haque and Ghosh 2013a; 2016b, c) where meiotic abnormalities are comparatively lower and pollen viability was higher in ex vitro plants than in in vivo plants. In the present study, we found increasing percentage of meiotic abnormalities in in vitro flowers, which led to a decrease in pollen viability, compared with in vivo plants. These findings are conflicting with those reported for *Momordica* and *Bacopa* (Wang et al. 2001; Haque and Ghosh 2013c), where pollen viability of both in vivo and in vitro flowers did not differ significantly. On the basis of the findings of previous and present studies, we can assume that the increase in meiotic abnormalities in in vitro flowers is mainly caused by the in vitro environment. However, the viability of in vitro pollen found in our study is sufficient for successful in vitro fertilisation for developing fruits. Furthermore, this in vitro system has provided an opportunity to cytologists to study the meiotic behaviour throughout the year without depending on the flowering season.

Conclusion

We have successfully developed an efficient protocol for the high-frequency in vitro flowering and fruiting of *Capsicum*. A single protocol applicable to different cultivars and species obviously has some impact, particularly when *Capsicum* is disreputed for its recalcitrant nature towards in vitro culture. Results indicated that BAP is the key factor in flower induction, while Ag^{+} ions increase the flowering percentage and promote normal development of floral buds. In addition, artificial hand pollination and supplementation of high concentration of sucrose facilitated fruit setting and ripening. Thus, the protocol described in this study offers a repeatable system for studying the physiological mechanism of flowering and fruiting.

Acknowledgments SMH acknowledges the Ministry of Minority Affairs (MOMA) and the University Grant Commission (UGC) of India for providing Maulana Azad National Fellowship (MANF). BG thanks the Department of Science and Technology, West Bengal (DST-WB) for providing a research Grant. All authors acknowledge Dr. Paul W. Bosland, Professor of the Department of Agronomy and Horticulture, New Mexico State University, Mexico for gifting us the seeds of Mexican cultivars of *Capsicum*, and Swami Kamalasthananda, Principal, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata, India for the facilities provided during the present study. The DST-FIST programme for infrastructural facilities is also acknowledged.

Authors' contribution SMH carried out all the experimental works and statistical analysis under the guidance of BG. SP help to SMH during experimental work. The manuscript was initially drafted by SMH and critically revised by BG, then finally checked and approved by all authors. BG obtained a research grant [Grant Number 616(Sanc.)/ST/P/S&T/1G-6/2011] for conducting the work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Aloni B, Karni L, Rylski I (1995) Inhibition of heat induced pepper (*Capsicum annuum*) flower abscission and induction of fruit malformation by silver thiosulphate. J Hortic Sci 70:215–220. doi:10.1080/14620316.1995.11515290

Bais HP, Sudha GS, Ravishankar GA (2000) Putrescine and $AgNO_3$ influences shoot multiplication, in vitro flowering and endogenous titers of polyamines in *Cichorium intybus* L. cv. Lucknow local. J Plant Growth Regul 19:238–248. doi:10.1007/s003440000012

Bakshi A, Shemansky JM, Chang C, Binder BM (2015) History of research on the plant hormone ethylene. J Plant Growth Regul 34:809–827. doi:10.1007/s00344-015-9522-9

Beyer EM (1976) A potent inhibitor of ethylene action in plants. Plant Physiol 58:268–271

Bodhipadma K, Leung DWM (2003) In vitro fruiting and seed set of *Capsicum annuum* l. cv. Sweet Banana. In Vitro Cell Dev Biol-Plant 39:536–539. doi:10.1079/IVP2003441

da Silva JAT, Kerbauy GB, Zeng S, Chen Z, Duan J (2014) In vitro flowering of orchids. Crit Rev Biotechnol 34(1):56–76. doi:10.3109/07388551.2013.807219

Das A, Kundu S, Ghosh B (2015) A simple and efficient method for extraction and quantification of capsaicin from pepper fruits through high performance thin layer chromatography. RJPBCS 6(1):606–612. http://www.rjpbcs.com/pdf/2015_6(1)/[73].pdf

De Jeu MJ (2000) In vitro techniques for ornamental breeding. Acta Hortic 508(508):55–60. doi:10.17660/ActaHortic.2000.508.6

Franklin G, Pius PK, Ignacimuthu S (2000) Factors affecting in vitro flowering and fruiting of green pea (*Pisum sativum* L.). Euphytica 115:65–73. doi:10.1023/A:1003982900117

Haque SM, Ghosh B (2013a) High frequency microcloning of *Aloe vera* and their true-to-type conformity by molecular cytogenetic assessment of two years old field growing regenerated plants. Bot Stud 54:46. http://www.as-botanicalstudies.com/content/54/1/46

Haque SM, Ghosh B (2013b) In vitro completion of sexual life cycle: production of R_1 plants of *Ipomoea quamoclit* L. Propag Ornam Plants 13:19–24. http://www.journal-pop.org/2013_13_1_19-24.html

Haque SM, Ghosh B (2013c) Micropropagation, in vitro flowering and cytological studies of *Bacopa chamaedryoides*, an ethnomedicinal plant. Environ Exp Biol 11:59–68. http://eeb.lu.lv/EEB/201303/EEB_11_Haque.pdf

Haque SM, Ghosh B (2016a) Cytological studies of sporophytic and gametophytic generation of two bulbaceous species *Ledebouria revoluta* and *Drimiopsis botryoides* (Asparagaceae). Caryologia 69:38–49. doi:10.1080/00087114.2015.1109940

Haque SM, Ghosh B (2016b) Cell division study in 'pollen mother cells' and 'pollen grains' of in vivo and ex vitro plants in *Drimiopsis botryoides*. Grana. doi:10.1080/00173134.2016.1144785

Haque SM, Ghosh B (2016c) High-frequency somatic embryogenesis and artificial seeds for mass production of true-to-type plants in *Ledebouria revoluta*: an important cardioprotective plant. Plant Cell Tissue Organ Cult. doi:10.1007/s11240-016-1030-5

Hoyer L (1998) Silver thiosulphate can considerably reduce leaf and fruit abscission caused by ethylene in *Capsicum annuum* 'Janne'. J Hortic Sci Biotechnol 73:29–34. doi:10.1080/14620316.1998.11510939

Jeong BR, Sivanesan I (2015) Direct adventitious shoot regeneration, in vitro flowering, fruiting, secondary metabolite content and antioxidant activity of *Scrophularia takesimensis* Nakai. Plant Cell Tissue Organ Cult 123:607–618. doi:10.1007/s11240-015-0864-6

Joshi M, Nadgauda RS (1997) Cytokinins and in vitro induction of flowering in bamboo: *Bambusa arundinacea* (Retz.) Willd. Curr Sci 73:523–526

Kamvorn W, Techawongstien S, Techawongstien S, Theerakulpisut P (2014) Compatibility of inter-specific crosses between *Capsicum chinense* Jacq. and *Capsicum baccatum* L. at different fertilization stages. Sci Hortic 179:9–15. doi:10.1016/j.scienta.2014.09.003

Kaur D, Dogra V, Thapa P, Bhattacharya A, Sood A (2015) In vitro flowering associated protein changes in *Dendrocalamus hamiltonii*. Proteomics 15:1291–1306. doi:10.1002/pmic.201400049

Kehie M, Kumaria S, Tandon P (2012) In vitro plantlet regeneration from nodal segments and shoot tips of *Capsicum chinense* Jacq. cv. Naga King Chili. 3. Biotech 2:31–35. doi:10.1007/s13205-011-0025-5

Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N (2010) Chilli peppers: a review on tissue culture and transgenesis. Biotechnol Adv 28:35–48. doi:10.1016/j.biotechadv.2009.08.005

Kumar V, Parvatam G, Ravishankar GA (2009) $AgNO_3$ - a potential regulator of ethylene activity and plant growth modulator. Electron J Biotechnol 12:2. doi:10.2225/vol12-issue2-fulltext-1

Kundu S, Das A, Ghosh B (2015a) Modulation of pungency and major bioactive compounds in pepper due to agro-climatic discrepancy: a case study with *Capsicum chinense* Bhut Jolokia fruit. Int J Pharm Pharm Sci 7:294–298

Kundu S, Das A, Haque SM, Ghosh B (2015b) Chemotypic diversity in different Bhut Jolokia fruits: in vitro conservation and mass propagation of superior ecotype. Int J Pharm Sci Rev Res 34(2):47–53

Manzur JP, Fita A, Prohens J, Rodríguez-Burruezo A (2015) Successful wide hybridization and introgression breeding in a diverse set of common peppers (*Capsicum annuum*) using different cultivated Ají (*C. baccatum*) accessions as donor parents. PLoS One. doi:10.1371/journal.pone.0144142

Máthé Á, Hassan F, Kader AA (2015) In vitro micropropagation of medicinal and aromatic plants. In: Máthé Á (ed) Medicinal and aromatic plants of the world. Springer, Berlin. doi:10.1007/978-94-017-9810-5_15

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Murthy KSR, Kondamudi R, Rao PVC, Pullaiah T (2012) In vitro flowering: a review. J Agric Technol. 8(5):1517–1536. http://www.ijat-aatsea.com/pdf/v8_n5_12_July/01_IJAT_2012_8(5)

Ochoa-Alejo N, Ramirez-Malagon R (2001) In vitro chili pepper biotechnology. In Vitro Cell Dev Biol-Plant 37:701–729

Paul S, Das A, Sarkar NC, Ghosh B (2013) Collection of chilli genetic resources from different geographical regions of West Bengal, India. Int J Bioresour Stress Manag 4:147–153. http://www.indianjournals.com/ijor.aspx?target=ijor:ijbsm&volume=4&issue=2&article=007

Ramchiary N, Kehie M, Brahma V, Kumaria S, Tandon P (2014) Application of genetics and genomics towards *Capsicum* translational research. Plant Biotechnol Rep 8:101–123. doi:10.1007/s11816-013-0306-z

Ribalta FM, Croser JS, Erskine W, Finnegan PM, Lulsdorf MM, Ochatt SJ (2014) Antigibberellin-induced reduction of internode length favors in vitro flowering and seed-set in different pea genotypes. Biol Plant 58(1):39–46. doi:10.1007/s10535-013-0379-0

Sarker RH, Das SK, Hoque MI (2012) In vitro flowering and seed formation in lentil (*Lens culinaris* Medik.). In Vitro Cell Dev Biol-Plant 48:446–452. doi:10.1007/s11627-012-9444-1

Sharma A, Kumar V, Giridhar P, Ravishankar GA (2008) Induction of in vitro flowering in *Capsicum frutescens* under the influence of silver nitrate and cobalt chloride and pollen transformation. Electron J Biotechnol 11:2. doi:10.2225/vol11-issue2-fulltext-8

Sheeja TE, Mandal AB (2003) In vitro flowering and fruiting in tomato (*Lycopersicon esculentum* Mill.). Asia Pac J Mol Biol Biotechnol 11(1):37–42. http://www.msmbb.org.my/apjmbb/html111/pg37.pdf

Sim GE, Loh CS, Goh CJ (2007) High frequency early in vitro flowering of *Dendrobium* Madame Thong-In (Orchidaceae). Plant Cell Rep 26:383–393. doi:10.1007/s00299-006-0242-2

Sivanandan G, Theboral J, Dev GK, Selvaraj N, Manickavasagam M, Ganarathi A (2015) Effect of carbon and nitrogen sources on in vitro flower and fruit formation and withanolides production in *Withania somnifera* (L.) Dunal. Indian J Exp Biol 53:177–183. http://www.ncbi.nlm.nih.gov/pubmed/25872249

Sivanesan I, Park SW (2015) Optimizing factors affecting adventitious shoot regeneration, in vitro flowering and fruiting of *Withania somnifera* (L.) Dunal. Ind Crop Prod 76:223–328. doi:10.1016/j.indcrop.2015.05.014

Taylor NJ, Light ME, Staden JV (2005) In vitro flowering of *Kniphofia leucocephala*: influence of cytokinins. Plant Cell Tissue Organ Cult 83:327–333. doi:10.1007/s11240-005-8429-8

Thul ST, Lal RK, Shasany AK, Darokar MP, Gupta MM, Verma RK, Khanuja SPS (2009) Estimation of phenotypic divergence in a collection of *Capsicum* species for yield-related traits. Euphytica 168:189–196. doi:10.1007/s10681-009-9882-y

Tisserat B, Galletta PD (1995) In vitro flowering and fruiting of *Capsicum fruitescens* L. HortScience 30(1):130–132. http://hortsci.ashspublications.org/content/30/1/130.full.pdf+html

Todorović SD, Grubišić DZ, Giba ZD, Mišić Konjević R (2006) Sucrose effects on in vitro fruiting and seed production of *Centaurium pulchellum*. Biol Plant 50(4):771–774. doi:10.1007/s10535-006-0128-8

Tomiczak K, Mikuła A, Sliwinska E, Rybczyński JJ (2015) Autotetraploid plant regeneration by indirect somatic embryogenesis from leaf mesophyll protoplasts of diploid *Gentiana decumbens* L.f. In Vitro Cell Dev Biol-Plant 51:350–359. doi:10.1007/s11627-015-9674-0

Wang S, Tang L, Chen F (2001) In vitro flowering of bitter melon. Plant Cell Rep 20:393–397. doi:10.1007/s002990100351

Würschum T (2015) Ethylene inhibitors improve efficiency of microspore embryogenesis in hexaploid triticale. Plant Cell Tissue Organ Cult 122:751–757. doi:10.1007/s11240-015-0808-1

Zhang T (2007) In vitro flowering of *Perilla frutescens*. In Vitro Cell Dev Biol-Plant 43:91–94. doi:10.1007/s11627-007-9038-5