

EXPLORING EMBRYOGENIC COMPETENCE IN ANTHERS OF BITTER GOURD (*MOMORDICA CHARANTIA* L.) CULTIVAR FAISALABAD LONG

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ABSTRACT

Haploid plant development through androgenesis is highly genotype dependent. Hence, native high yielding bitter gourd cultivar Faisalabad long was explored for androgenesis and development of embryogenic calli from somatic tissues. Flower buds of different sizes (small 11-13 mm; medium 13-15 mm; large 15-17 mm in length) at popcorn stage were collected. The floral buds ranging from 13-15 mm in size higher percentage of uninucleate microspores compared with other sizes. Cold pretreatment of anthers at 4°C up to 48 hours and cultures under dark conditions significantly enhanced callus induction (71% and 45%) response compared with cultures under long days on MS media supplemented with NAA and BAP (3.22+0.88 μM). Rest of the PGRs used did not induce calli. Anther derived calli showed development of embryogenic masses upon transfer to starvation media ($\frac{1}{2}$ MS salts), however the embryos did not develop further and could not germinate. Hence, further studies are suggested for better embryo germination and maturation.

Key words: anthers, callogenesis, NAA, bitter melon.

INTRODUCTION

Hybridization programs are based on inbred or pure parental lines for developing hybrids of interest. Conventional breeding methods need several generations to develop nearly homozygous lines. Alternate techniques involve development of haploids using anther culture, microspore culture and ovule culture. These techniques shorten the breeding cycle, ensures fast track complete homozygosity and use of plant material for further genetic improvements (Dunwell, 2010; Germana, 2011). Many factors influence androgenesis, including plant genotype, physiological state of the parent plant, microspore development stage, temperature pretreatment of flower buds and the culture medium (Bajaj, 1990). In cucurbitaceae, androgenesis has been reported in squashes, muskmelon and cucumber (Lazarte and Sasser, 1982; Dryanovska, 1985; Metwally *et al.*, 1998). Bitter gourd (BG) also called as bitter melon, bitter apple or karela (*Momordica charantia* L.) is one of most popular cucurbitaceous vegetable commonly cultivated in South East Asia and China (Krawinkel and Keding, 2006). The genus *Momordica* includes 45 species of climbing herbaceous plants native to South Asia. These are grown for their fleshy fruits having diverse shapes, colors and skin. The axillary flowers contain androecium comprising 3 stamens (Kumar *et al.*, 2010). BG ranks first among cucurbits for iron (0.43 mg/100 gm FW) and vitamin C availability. It is known to treat diabetes, cancer and other major diseases (Fang *et al.*, 2011). In Pakistan, pumpkins, squashes and gourds are produced as merely 9.92 metric tons/ha compared with China (18.5 metric tons/ha) and

average of Asian countries 13.7 metric tons/ha, respectively (FAOSTAT, 2011). This huge yield gap in Pakistan is due the absence of high yielding hybrids better adapted to local environment suggesting dire need to initiate indigenous crop improvement programs. Production of haploids in BG through anther culture would allow breeders for an efficient release of pure parental lines and better screening for resistance to diseases. Since genotype and explant age play a critical role in androgenesis (Murovec and Bohanec, 2012), high yielding native BG cultivar Faisalabad long (FL) was selected to establish androgenesis system for future crop improvement applications. Cultivar FL is good quality, long fruit bearing and resistant against melon fruit fly (Hussain, 1990; Gogi *et al.*, 2010). Further, cold treatment is known to enhance microspore embryogenesis in *Brassica napus* and *B. oleracea* anthers inside the buds (Gu *et al.*, 2004; Yuan *et al.*, 2011). Hence, the current study was initiated to explore anthers of cultivar Faisalabad long for their genotypic potential to develop embryogenic calli on different media and culture conditions.

MATERIALS AND METHODS

Plant materials, anther collection and fixation: Seeds of elite indigenous BG cultivar Faisalabad long were taken from Ayub Agriculture Research Institute (AARI), Faisalabad. The crop was raised in vegetable research area of the Horticulture Institute, UAF as source of anthers. Flower buds of different sizes (small 11-13 mm; medium 13-15 mm; large 15-17 mm in length) at popcorn

stage were collected from the donor plant population at 8-10 am in the morning. The buds were counted for no. of anthers and anther length under different sizes after removal of petals (Fig. 2A). For cytological studies, buds were kept in fixative solution (alcohol: acetic acid 3:1) for upto 12 h and preserved in 70% ethanol solution (v/v). The anthers were excised from these buds, crushed over a microscope glass slide by applying gentle pressure on the cover slide. Slides were observed under the Nikon Optiphot Fluorescence Microscope at 40x for the uni-nucleate stage of the microspores (Summers *et al.*, 1992). The buds containing more percentage of anther with early to late uni-nucleate stage microspores were selected and stored moistened in plastic bags for cold pretreatment at 4°C for different time intervals (0, 24, 48, 72, 96 hrs) in dark (Fig. 2B).

Explant sterilization and culture conditions: After treatment, the buds were surface disinfected with 70% ethanol (v/v) + 1-2 drops of Tween-20 detergent for 2-3 minutes followed by 2-3 rinses with sterile water. Then the flower buds were sterilized in 5% sodium hypochlorite (v/v) solution for 5 minutes followed by 3 rinses with sterile water. Petals were removed aseptically and anthers were carefully excised with forceps and placed on MS (Murashige and Skoog, 1962) media modified with different auxins and cytokinin either alone or in combination for androgenesis called as MSA media (Table 1). Sucrose (30 gL⁻¹) was added as carbon source. Medium pH was adjusted at 5.7 and 8 g of agar (Phytotech, USA) was added as a solidifying agent in the media. Media were sterilized using autoclave for 20 minutes at 121 ± 1°C and 15 psi. Thirty anthers were cultured per treatment and calli induced were sub-cultured to MSO (control) and starvation media (½ MS salts, ¼ MS salts) for regeneration. Cultures were placed under long day (LDs: 16 h light: 8 h dark) and dark conditions in the growth room facilitated with 60-70 µEm⁻² sec⁻¹ light intensity using white fluorescent light and maintained at temperature 25 ± 1°C.

Statistical analysis: Experiments were replicated twice with at least 30 test tubes per treatment containing one explant per tube. These experiments were laid out according to Completely Randomized Design (CRD) and data were analyzed using Genstat software (12th Ed.) and Least Significant Difference (LSD) test was used for estimation of significant differences between individual treatments (Steel *et al.*, 1997).

RESULTS

Medium sized buds produced more anthers at desired stage for androgenesis: Significant differences were observed for number of developed and under-developed

anthers under 13-15 mm and 15-17 mm anther sizes compared with 11-13 mm size (Fig. 1a). Anther length was significantly higher in 15-17 mm long buds in developed and under-developed anthers compared with other categories (Fig. 1b). Cytological analysis of buds of different length revealed higher percentage > 80% of uni-nucleate to early bi-nucleate microspores in medium sized (13-15 mm) buds compared with other categories. Hence these buds were selected as anther source.

Dark conditions enhanced androgenic response compared to Long Days (LDs): Anthers cultured on different types of androgenesis media induced calli only on MS media supplemented with different levels of NAA+BAP. Callus induction response peaked at low levels of NAA+BAP (1.07+0.88 µML⁻¹ and 2.14+0.88 µML⁻¹) under Dark (61%-71%) compared with LDs conditions (49%-61%) as shown in Fig. 3. A later peak was observed for callus induction under LDs (3.22+0.88 µML⁻¹) that was statistically non-significant to calli induced under D conditions. Taken together, calli induction was early and higher under D compared with LDs conditions (Fig. 3). Both callus induction and proliferation rate was higher at 3.22+0.88 µML⁻¹ of NAA+BAP compared with other treatments and calli were greenish yellow in color, compact and friable in texture (Fig., 2C; Table 2).

Cold pre-treatment improved androgenic response: Anther pretreatment at 4°C significantly enhanced callus induction response (40-45%) compared with control (26%) (Fig. 4). Cold treatment up to 48 h showed maximum callus induction response and further increase in the treatment time did not enhance callus induction suggesting 48 h as the optimal interval for low temperature treatment for BG anthers. Callus induction response remained consistently higher throughout treatments on MSA media at 3.22+0.88µML⁻¹ of NAA+BAP compared with control and other PGR treatment levels suggesting it as the optimal callus induction level (Fig. 4). Consistent to previous results higher callus induction response (61%-63%) was obtained at 3.22+0.88 µML⁻¹ levels of NAA+BAP independent of culture conditions and pre-treatment suggesting it as the optimal level for callus induction (Fig. 5).

The induced calli were sub-cultured to MSO and starvation media to induce embryogenesis. The calli showed development of embryogenic masses on starvation media (½MS media) after 3 weeks of culture on surface of the calli, however, no further embryo development could be induced after 8-10 weeks of subcultures. These findings suggest that the pseudo embryo like structures may be induced that could not grow and germinate.

Table 1. Media composition for androgenesis (MSA media)

Treatments	MS media + PGRs (μML^{-1})				
	2,4-D	NAA	BAP	NAA + BAP	BAP + NAA
T ₀	0	0	0	0	0
T ₁	0.90	1.07	0.88	1.07+0.88	0.88+1.07
T ₂	1.81	2.14	1.77	2.14+0.88	1.77+1.07
T ₃	2.71	3.22	2.66	3.22+0.88	2.66+ 1.07
T ₄	3.62	4.29	3.55	4.29+0.88	3.55+1.07

Table 2. Morphological characterization of anther derived calli on NAA and BAP

MS media + PGRs (μML^{-1})	CI	CP	Calli color
Control	-	-	-
1.07+0.88	++	+	Light green
2.14+0.88	++	++	Light green
3.22+0.88	+++	++++	Greenish yellow
4.29+0.88	++	++	Greenish yellow

Poor (-)

Normal (+)

Good (++)

Better (+++)

Excellent (++++)

CI callus induction

CP callus proliferation

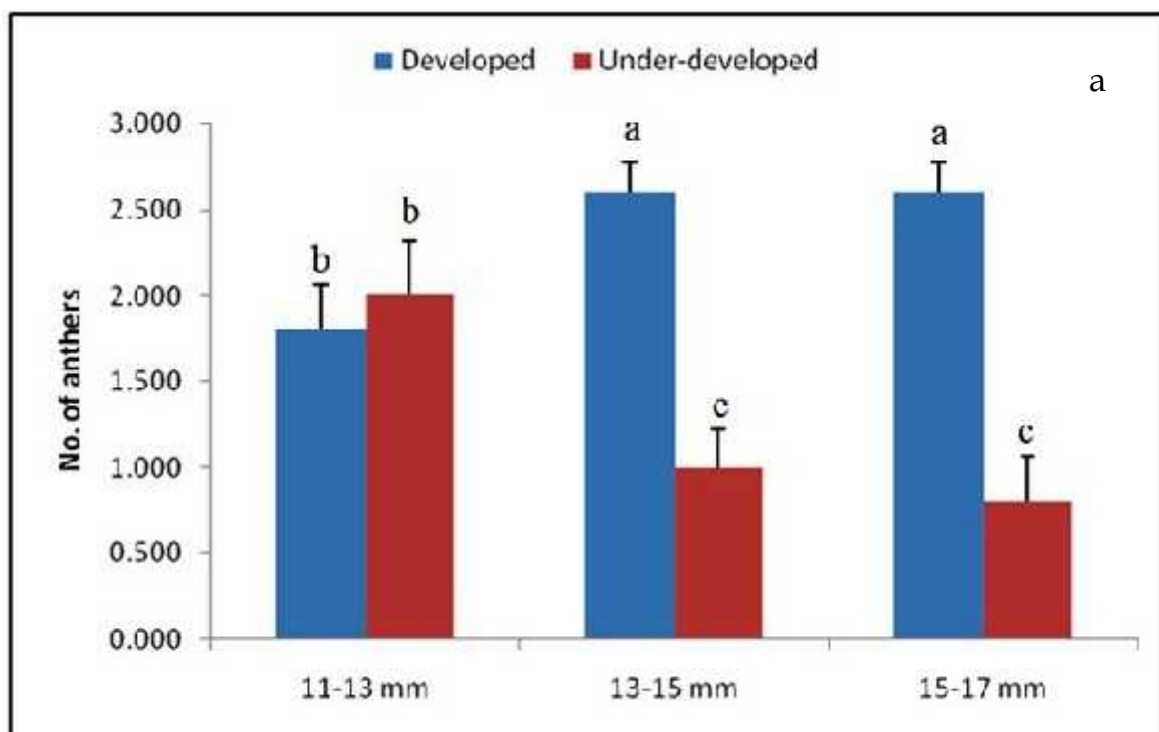


Fig. 1. Anther number (a) and size (b) in relation to unopened floral bud length in Bitter melon cv. Faisalabad long. Values are means of number of anthers and anther length per bud, where $n = 10$. Error bars indicate SE. The vertical bars sharing different letters show significant difference between the calculated ($P < 0.05$) values.

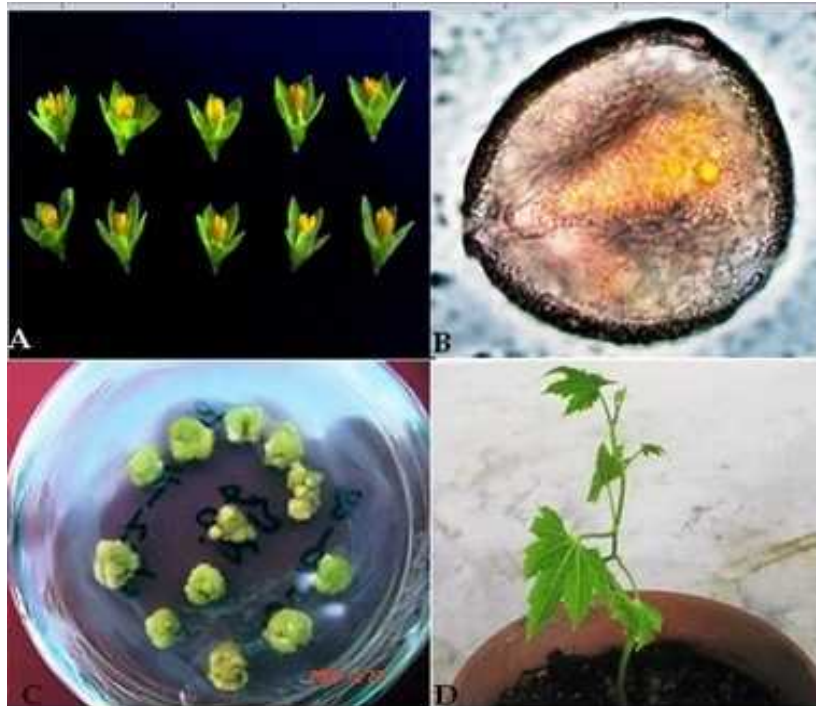
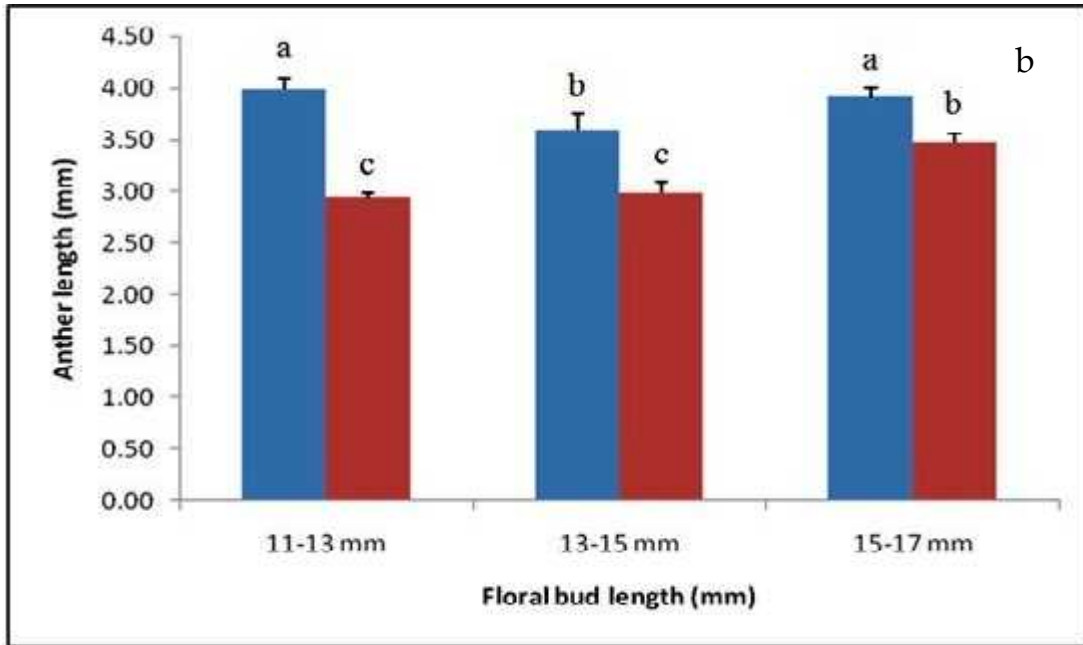


Fig. 2. Callogenic response of anthers and leaf disc in Bitter gourd cv. Faisalabad Long. The figures show A) medium sized floral buds after petal removal, B) fully developed vacuolated microspore, C) androgenic calli induced on MS media supplemented with NAA + BAP and D) in vitro propagated plants of Bitter gourd from stem cuttings in compost plus sand media after acclimatization.

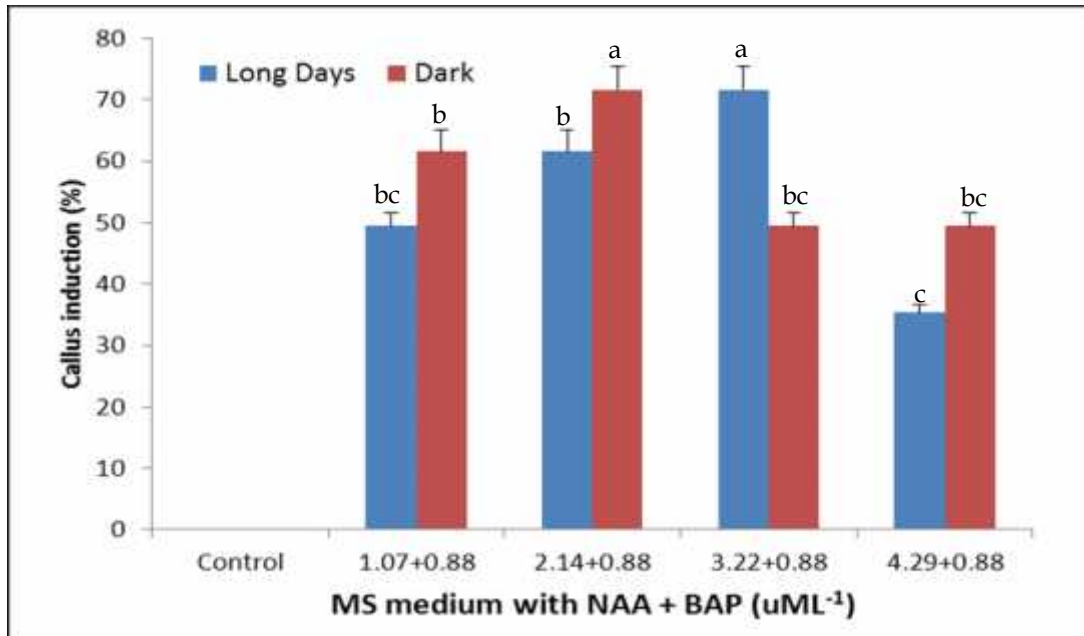


Fig. 3. Photomorphogenic response in Bitter gourd cv. Faisalabad long anthers for callus induction on MS medium supplemented with NAA+BAP (μML^{-1}). Values are based on the analysis of calli induced after 4 weeks of culture in vitro, where $n = 30$. Error bars indicate SE. The vertical bars sharing different letters show significant difference between the calculated ($P < 0.05$) values for callus induction compared with control.

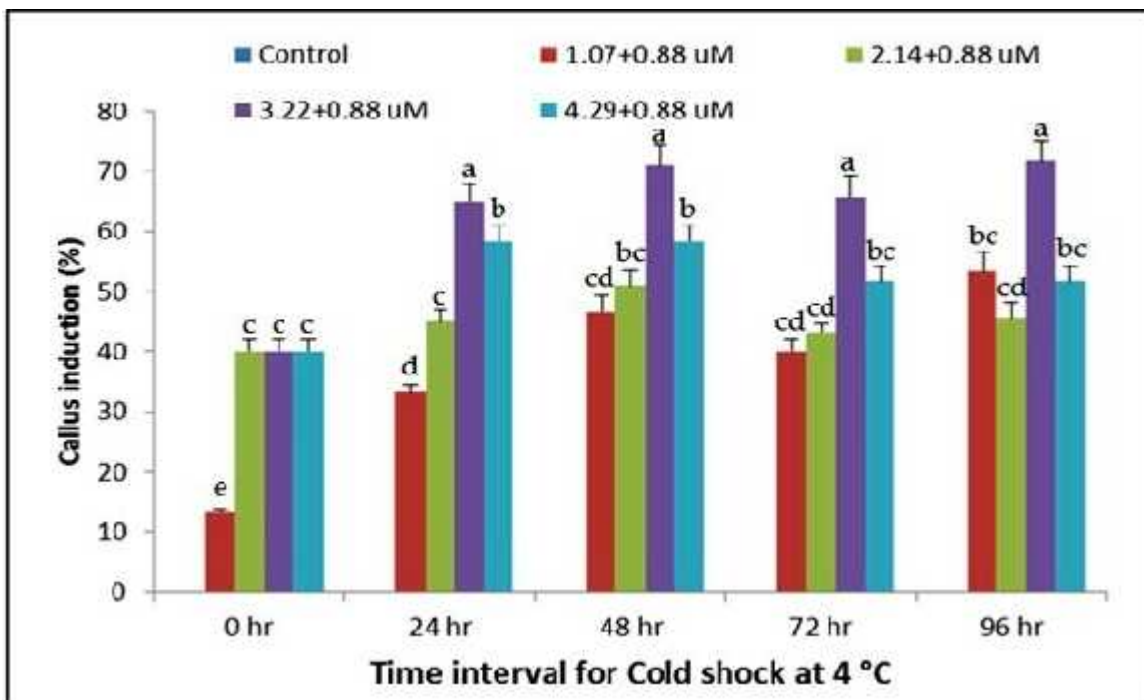


Fig. 4. Effect of cold shock pre-treatment in Bitter gourd cv. Faisalabad long anthers at 4°C for 0-96 hrs time intervals for callus induction on MS medium supplemented with NAA+BAP (μML^{-1}). Values are based on the analysis of calli induced after 4 weeks of culture in vitro, where $n = 30$. Error bars indicate SE. The vertical bars sharing different letters show significant difference between the calculated ($P < 0.05$) values for callus induction compared with control.

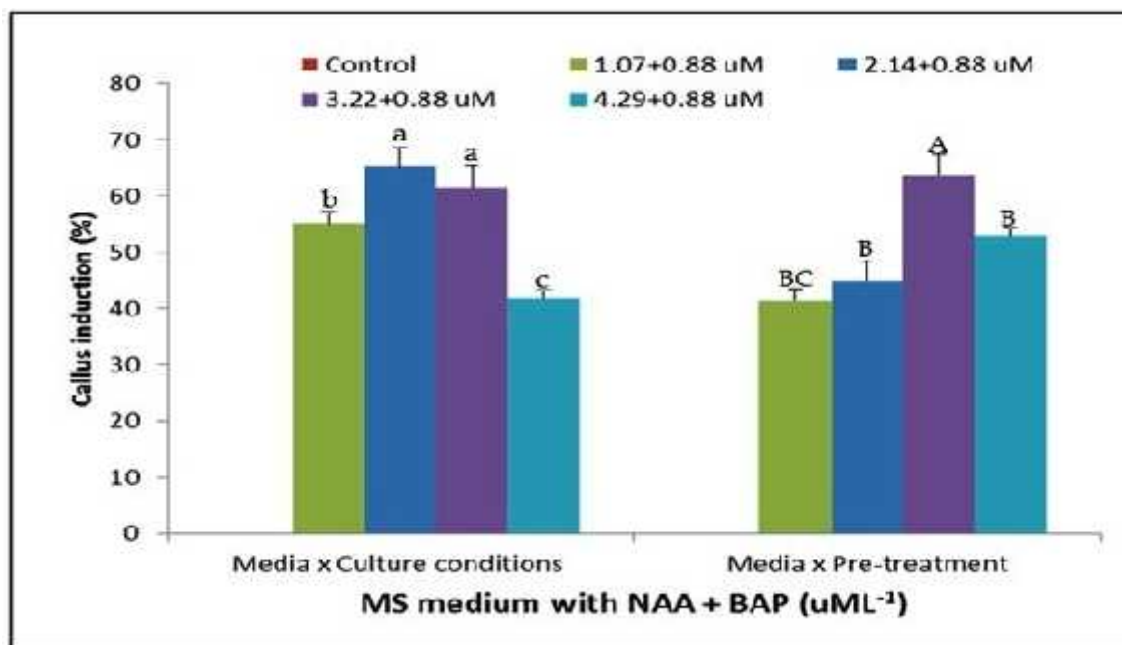


Fig. 5. Interactive response of pre-treatment (cold shock at 4°C) and culture conditions (light/dark for callus induction on MS medium supplemented with NAA+BAP (μML^{-1}) in Bitter gourd cv. Faisalabad long anthers . Values are based on the analysis of calli induced after 4 weeks of culture in vitro, where $n = 30$. Error bars indicate SE. The vertical bars sharing different small/capital letters show significant difference between the calculated ($P < 0.05$) values for callus induction compared with control.

DISCUSSION

Pure line development through classical breeding needs time and resources. Androgenesis has enormous potential to produce double haploid lines through microspore or pollen derived embryos or calli (Segui-Simarro *et al.*, 2011) and can be induced in both monocots and dicots (Dunwell, 2010). Haploids can be regenerated from anther or microspore explants and genome can be doubled using genome doubling agents like colchicine. The doubled haploid plants will be 100% homozygous and could be used as parental lines in hybridization programs and a better plant material for genome mapping studies. In more than 250 crops including herbaceous cereals to perennial tree crops, haploid plant development protocols are available (Segui-Simarro *et al.*, 2011). However, except few model crops, efficiency of plant regeneration is still quite low suggesting further fine tuning the technology. One of the most critical reasons of low efficiency is that optimized protocols are not equally functional for all the genotypes available under different geographic localities in a crop (Corral-Martinez *et al.*, 2010). Other physical and environmental cues like light conditions, media, stress and temperature also play an important role in induction of androgenesis as reported in eggplant and other crop sp. (Rotino *et al.*, 2005; Karami and Saidi, 2010). Hence, BG

cultivar Faisalabad long was explored for androgenesis under long days and dark culture conditions and using cold pretreatment of anthers. Consistent to previous finding, we obtained higher calli induction and proliferation response on MS media supplemented with NAA and BAP. Cold treatment at 4°C for upto 48 h and dark cultures significantly enhanced calli growth compared with cultures under long days (LDs). Similar effects of cold treatment enhancing calli induction are reported in oil seed rape and broccoli (Gu *et al.*, 2004; Yuan *et al.*, 2011). The calli when transferred to starvation media showed embryogenic masses that did not regenerate into plants and developed non-functional zygote-like shoot apical meristem (SAM). Similar response was reported in other crops where neither calli nor embryos showed plant regeneration like eggplant (Bal *et al.*, 2009) and pepper where plant regeneration was as low as 0.1 per bud from microspore culture (Regner, 1996). The problems in obtaining embryos from anthers and microspore culture are also confirmed by Parra-Vega *et al.* (2010) in sweet pepper as some of the embryos developed in to undifferentiated callus like growth similar to report in eggplant as well. In another report, calli were induced in Chinese bitter melon on media supplemented with higher concentration of kinetin and AgNO_3 , however, calli did not regenerate in to embryos (Tang *et al.*, 2012). We differ in media

composition, pH, agar % and growth conditions provided for androgenesis. Consistent to our results, dark cultures also enhanced androgenic response in carrot anthers (Gorecka *et al.*, 2005). In contrast, cold treatment declined androgenic competence of anthers in pepper (Ciner and Tipirdamaz, 2002). These findings suggest further digging out the deficiencies in the available techniques to enhance better transformation efficiency of embryogenic masses in to mature germinating embryos or induce better shoot primordia to induce shoot regeneration in the calli. Further, some cultivars show null response to one method while these may respond to the other (Malik *et al.*, 2008). There is need to further deepen our knowledge about the genotypic behavior of different cultivars to complex interactive effect of media, culture conditions and different stress types to enhance efficiency of haploid development.

Conclusions: We demonstrate here enhanced androgenic response in anthers of BG cultivar Faisalabad long in response to dark culture conditions and cold shock for 48 h. These results may contribute to our understanding in the process of better androgenic behavior from anthers that may be further useful for the transformation of important biomolecules in BG.

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