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LONG DAYS FOSTER CALLOGENESIS IN SPINACH AND LETTUCE CULTIVARS

M. Usman*, S. Noureen, B. Fatima and Q. Zaman

Institute of Horticultural Sciences, University of Agriculture, Faisalabad
*Corresponding author Email: musman74@gmail.com

ABSTRACT

Explants of different age were cultured on Murashige and Skoog (MS) medium containing auxins and cytokinins in elite leafy vegetables under different photoperiodic conditions to explore *in vitro* regeneration. Fifteen days old explants produced more calli in both crops compared with 7 days old explants. Though, dark culture conditions triggered more calli on lower levels of 2,4-D (0.5-2.0 mgL^{-1}) compared with higher levels in Cotyledon and Hypocotyl explants, however, calli growth was greater under long days in both crops. Similar better calli growth response was observed on BAP and Kinetin media in leaf disc explants in Lettuce under long days. These studies suggest that light stimulus may boost the endogenous hormonal production and crosstalk of endo and exogenous hormones trigger early calli growth and proliferation. Further studies are implied to analyze the endogenous hormonal regulation in response to photoperiodic changes in leafy vegetables. These studies could be further useful for expressing important biomolecules in the edible leafy vegetables and for exploring somaclonal variation.

Key words: Photomorphogenesis, light, dark, callus, leafy vegetables.

INTRODUCTION

Herbaceous plants harvested for their leaves are known as leafy vegetables including spinach, lettuce, cabbage, fenugreek and celery. Spinach (*Spinacia oleracea* L.) is native to Asia and is high value vegetable rich in vitamins, calcium, iron and fiber content in its leaves (Knoll *et al.,* 1997). China is leading in global spinach industry producing 85% of total world's production following by United States yielding 3% crop. Pakistan is producing 95.5 thousand tons of spinach with 1.2 $tonsha^{-1}$ yield. Lettuce (*Lactuca sativa* L.) is also another highly important leafy vegetable, family Compositeae, having salient medicinal properties. It is good dietary source of vitamins C, E, K, carotenoids and known as king of salad crops round the globe (Hunter and Burritt, 2002; Nicolle *et al.,* 2004). Lettuce production in Pakistan is merely 135 tons with 0.13 tons ha^{-1} yield (FAOSTAT, 2010). Like other vegetable crops, improvement in leafy vegetables is highly desired to meet future food demands and ensure food security. Development of efficient plant tissue culture protocols is basic requirement for genetic transformation to develop resistance against biotic and abiotic stresses and for cultivar improvement. Callus induction and shoot regeneration in both crops have been highly genotype dependent (Knoll *et al.,* 1997; Mohebodini *et al.,* 2011) and within cultivar variation is also reported in spinach like melon. A few spinach cultivars like Nippon and Longstanding have shown higher regeneration potential (Zhang and Zeevart, 1999; Ishizaki *et al.,* 2001).

Culture conditions play a significant role in *in vitro* plant regeneration as these strongly influence the morphogenesis in cultured cells and tissues. The culture medium, temperature, pH and humidity have been studied thoroughly, however, fewer attempts have been made to evaluate and optimize light requirements for growth and differentiation. Light quality controls numerous plant characteristics and is known to influence the growth and development of mature plants and morphogenesis (Lee *et al.,* 2007; Mengxi *et al.,*2011). Darkness may completely inhibit shoot induction for example from hypocotyl in tomato and leaves in *Petunia hybrida* (Lercari *et al.,* 1999; Reuveni and Evenor, 2007). Explant type may also play a critical role as root segments are reported superior for somatic embryogenesis in spinach and leaf disc for shoot induction in tomato and cucumber (Zhang and Zeevaarat, 1999; Khan *et al.* 2006; Usman *et al.* 2011). Thin cell layers from hypocotyls and roots have been utilized in organogenesis by Knoll *et al.* (1997). Differential *in vitro* regeneration and multiplication response of different genotypes signify the need to test potential indigenous and exotic cultivars of important leafy vegetables spinach and lettuce under long day (LD) and dark (D) culture conditions for regeneration.

MATERIALS AND METHODS

Media preparation and sterilization: Murashige and Skoog (MS; 1962) medium was used as basal medium for seed germination, plant multiplication and further regeneration of spinach and lettuce cultivars. Sucrose ($30gL^{-1}$) was added as carbon source. Medium pH was adjusted at 5.7 and 8 gm of agar was added as a solidifying agent in the media. The media were sterilized in an autoclave for 20 minutes at 121°C and 15 psi.

Seed sterilization and explant sources: Seeds of different indigenous 'Faisalabad (Fsd.) Local', 'Kandiari'; exotic spinach hybrid cultivars 'Philipino' (Fig. 1A,B) and exotic lettuce cultivars viz. 'Bergamo', 'Grand rapid', 'Red revolution' and 'Iceberg' were collected from Ayub Agriculture Research Institute, Faisalabad and other commercial sources. Seeds of both crops were surface disinfected with 70% ethanol (v/v) plus 1-2 drops of Tween-20 detergent for 2-3 minutes followed by 3-5 rinses with sterilized distilled water. These seeds were dipped in 5% sodium hypochlorite (v/v) for 3-5 minutes followed by 5 rinses in sterilized water. *In vitro* raised seedlings were micropropagated on MS media following standard procedures (Fig. 1C, E) and the plantlets were used as source for explants such as hypocotyl (Hyp), cotyledon (Cot) and leaf disc for callus induction and further regeneration studies. These explants were cultured on modified MS medium supplemented with different levels (0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mgL^{-1} each) of either auxin 2,4-Dichlorophenoxyacetic acid (2,4-D), cytokinins including Benzylaminopurine (BAP) or Kinetin (KIN) for direct and indirect regeneration.

Culture conditions: Cultures were placed under long days (LD: 16 hrs light: 8 hrs dark) and dark (D) conditions in the growth room facilitated with 60-70 $\mu Em^{-2}sec^{-1}$ light intensity using white fluorescent light and maintained at temperature 25 ± 1°C to observe response of explants for callogenesis and regeneration on modified media. Calli induced on different media were sub-cultured for proliferation on the respective media and then transferred to MSO medium for regeneration.

Statistical analysis: Experiments were replicated thrice with at least 20 test tubes per replication. Each treatment had one explant per tube. These experiments were laid out according to Completely Randomized Design (CRD) and data was analyzed using MSTAT-C and significance among treatment means were compared using Duncan′s Multiple Range (DMRT) test by Steel *et al*., (1997).

RESULTS

A. Spinach Cultivars

1. Differential behavior of spinach explants and cultivars for callus induction on 2,4-D in response to photoperiodic alterations and calli characterization: Explant age showed significant difference in response for callus induction and 15 days old explants induced more calli compared with 7 days old explants (Data shown for 15 days old explants only). Darkness promoted callus induction in Fsd. Local cultivar at lower 1 mgL^{-1} and higher 3 mgL^{-1} levels of 2,4-D in cotyledon (Cot) explants (Fig. 1D, 2A) and concentration of 1.5-2 mgL^{-1} showed less callogenesis under both LDs and D culture conditions in both cultivars (Fig. 2B). In contrast in hypocotyl (Hyp) explants, callus induction sharply peaked ($P<0.05$) at 1.5-2 mgL^{-1} of 2,4-D regardless of the photoperiodic conditions. Among cultivars, Cot explants were found more responsive for callus induction in cv. Faisalabad local while Hyp explants were better in cv. Philipino Hybrid. Calli induced from Cot and Hyp explants were divided in small chunks and sub-cultured on the same media under LDs and D culture conditions for further growth and proliferation. Hyp explants induced calli under D conditions on 1.5-3 mgL^{-1} of 2,4-D, however callus proliferation was better under LD conditions on 2 mgL^{-1} of 2,4-D (Table 1). In contrast, Cot explants showed more callus induction on 1-1.5 mgL^{-1} of 2,4-D regardless of culture conditions whereas callus proliferation was more under dark conditions on 1.0 mgL^{-1} of 2,4-D. In most of the treatments calli developed were white to creamy white in color, friable in texture and can be easily divided into separate chunks of cells.

B. Lettuce Cultivars

1. Long days promoted early callogenesis in cotyledon explants on 2,4-D: Explant age showed a significant impact in callus induction as higher callus induction was observed in all the cultivars when 15 days old Cot explants were used compared with 7 days old explants under both LDs and D culture conditions (Data shown for 15 days old explants only). Long days promoted callus induction in Lettuce cultivars and consistent higher callus induction response was observed at lower levels of 2,4-D (0.5-1.5 mgL^{-1}) under LDs compared with D conditions (Fig. 3A). Lettuce cultivar Red revolution performed well and induced maximum calli (100%) on the lowest level of 2,4-D (0.5 mgL^{-1}) regardless of photoperiodic alterations in culture conditions followed by cv. Bergamo. The lowest callus induction response was depicted by cv. Grand rapid under both types of culture conditions. Calli grown was higher under LD conditions compared with dark when placed on MSO medium. Though callus induction was greater in LDs and D conditions on 0.5 and 1 mgL^{-1} however, calli proliferation was higher on 1.5 mgL^{-1} of 2,4-D. Calli color ranged from yellow to dark yellow (Table 2).

2. Long days enhanced callus induction in leaf disc explants on cytokinins: Callus induction from leaf disc explants was found to be strongly dependent upon genotype and Cytokinin used. Long Days promoted callus induction in leaf disc explants and significantly ($P<0.05$) higher callus induction was observed at lower levels of BAP compared with cultures exposed to continuous D conditions in all genotypes (Fig. 1G, 3B). Higher callus induction was observed in cv. Grand Rapid, Red revolution and Bergamo (86.67% to 93.3%) at 1-2 mgL^{-1} of BAP. Increase in level of BAP from 3 mgL^{-1} showed decrease in callus induction in cvs. Bergamo,

Grand rapid and Red revolution under both LDs and D conditions (Fig. 3B). Minimum callus induction (6.67%) was noted in cv. Iceberg on higher level of BAP (5 mgL^{-1}). Similar response for callogenesis was found on KIN. White fluorescent light under LDs promoted early and higher callus induction on KIN compared with consistent dark conditions showing delayed callus induction at higher KIN levels. Maximum calli were induced in cvs. Grand rapid (73.33%) on 2 $mgL^{-1}$of KIN followed by cvs. Bergamo, Red revolution and Iceberg (60% and 53.33% respectively) on 1 and 3 mgL^{-1} of KIN respectively. Among treatments, higher callus induction (35.83%) was observed on 2 mgL^{-1} KIN while further increase in KIN significantly reduced callus induction (Fig. 3C). Though callus induction was greater on KIN 3 mgL^{-1} however, calli proliferation rate was higher on 1.5 $mgL^{-1}$of BAP and 3 $mgL^{-1}$of KIN. Calli were light yellow to dark yellow in color (Table 2).

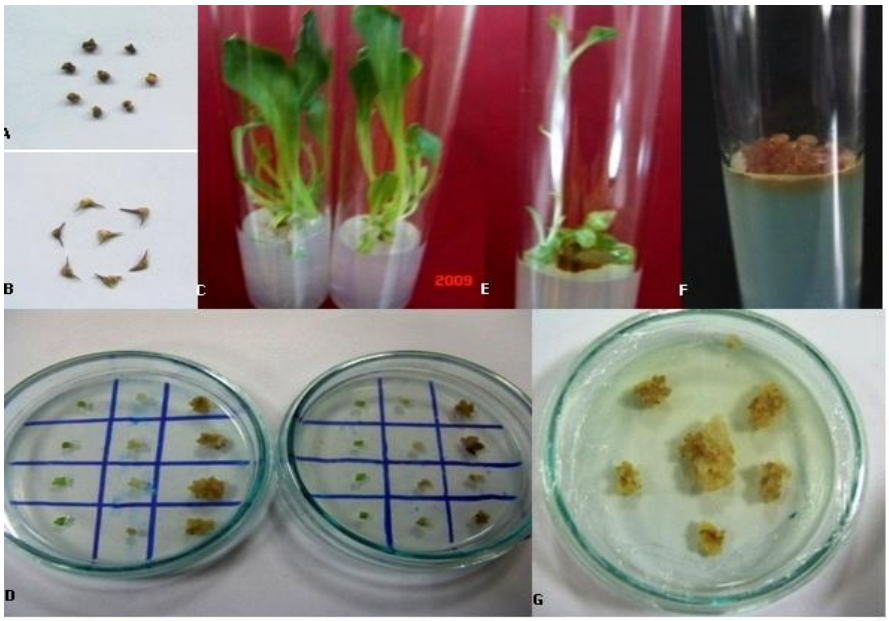

Fig. 1A-G. Response of explants for callus induction in spinach and lettuce genotypes. Where figs. A, B shows morphological differentiation in seeds of spinach cvs. Faisalabad local and Kandiari respectively; C) micropropagation of spinach cv. Faisalabad Local on MS medium; D) calli induced and proliferated in cotyledon leaf explant under dark condition on 2,4-D in cultivar Faisalabad Local; E) micropropagation of cv. Bergamo on MS medium; F) callus induction in 15 days old Cotyledon explant of lettuce cv. Red revolution under dark on 2,4-D (0.5mgL^{-1}); G) leaf disc explant induced calli proliferating on MS medium + BAP (1.5 mgL^{-1}) in cv. Iceberg under LD conditions

Table 1.Calli growth response in different explants on 2,4-D under Long day and dark culture conditions in spinach

MS medium + 2,4-D (mgL^{-1})	Hypocotyl explant				Cotyledon leaf explants			
	Callus induction		Callus proliferation		Callus induction		Callus proliferation	
	LD	D	LD	D	LD	D	LD	D
Control	-	-	-	-	-	-	-	-
0.5	+	+ +	+	+	-	+	-	+
1.0	+	+	+	+	+ + + + +	+ + + +	+ +	+ + +
1.5	+ +	+ + +	+ +	+	+ + + +	+ +	+ +	+ +
2.0	+ + +	++	+ + +	+ +	+ +	+	+	+
3.0	+	+ + + +	+	+	+ +	+ +	+	+
4.0	+	+	+	-	+	+	-	+
5.0	-	-	-	-	-	-	-	-

Data are taken from three reps. from two independent experiments with 20 explants per rep.
Poor (-), Normal (+), Good (++), Better (+++), Excellent (++++)
LD: Long days; D: Dark

Table 2. Calli growth response in different explants on 2,4-D, BAP and KIN under Long day and dark culture conditions in lettuce.

	2,4-D				BAP				KIN			
	Callus induction		Callus proliferation		Callus induction		Callus proliferation		Callus induction		Callus proliferation	
	LD	D	LD	D	LD	D	LD	D	LD	D	LD	D
Control	-	-	-	-	-	-	-	-	-	-	-	-
0.5	+ + +	+ + + +	+	+	++++	++++	+++	+	+ +	+ +	+ +	+
1.0	+ + + +	+ + +	+ + +	+ +	+++	+++	+++	+	+ + +	+ + +	+ +	+
1.5	+ + +	+ +	+ + + +	+	+++	+++	++++	+++	+ +	+ +	+ +	+
2.0	+ +	+	+ +	+	+++	+++	+ +	+ +	+ + +	+ + +	+ + +	+ +
3.0	+	+	+	+	+++	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+ +
4.0	+	-	-	-	++	+ +	+	+	++	+	+	+
5.0	+	-	-	-	+	+	-	+	+ +	+	-	+

Data are taken from three reps. from two independent experiments with 20 explants per rep.
Poor (-), Normal (+), Good (++), Better (+++), Excellent (++++)
LD: Long days; D: Dark

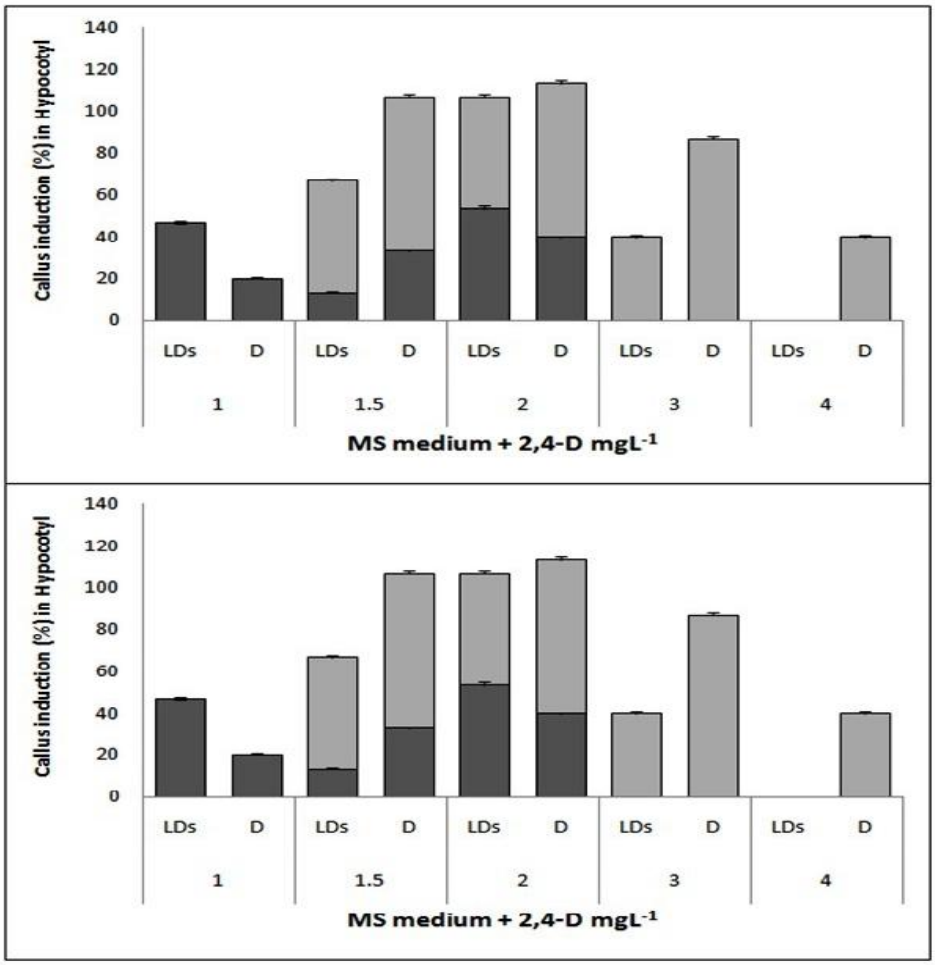

Fig. 2AB. Genotype and explant dependent callus induction (%) on MS medium containing 2,4-D in spinach under Long Days (LDs) and Dark (D) culture conditions. Data are means of 3 replicates plus standard error (SE) bars from two independent experiments with 20 explants per rep. Results are significantly different from each other at probability ($P<0.05$).

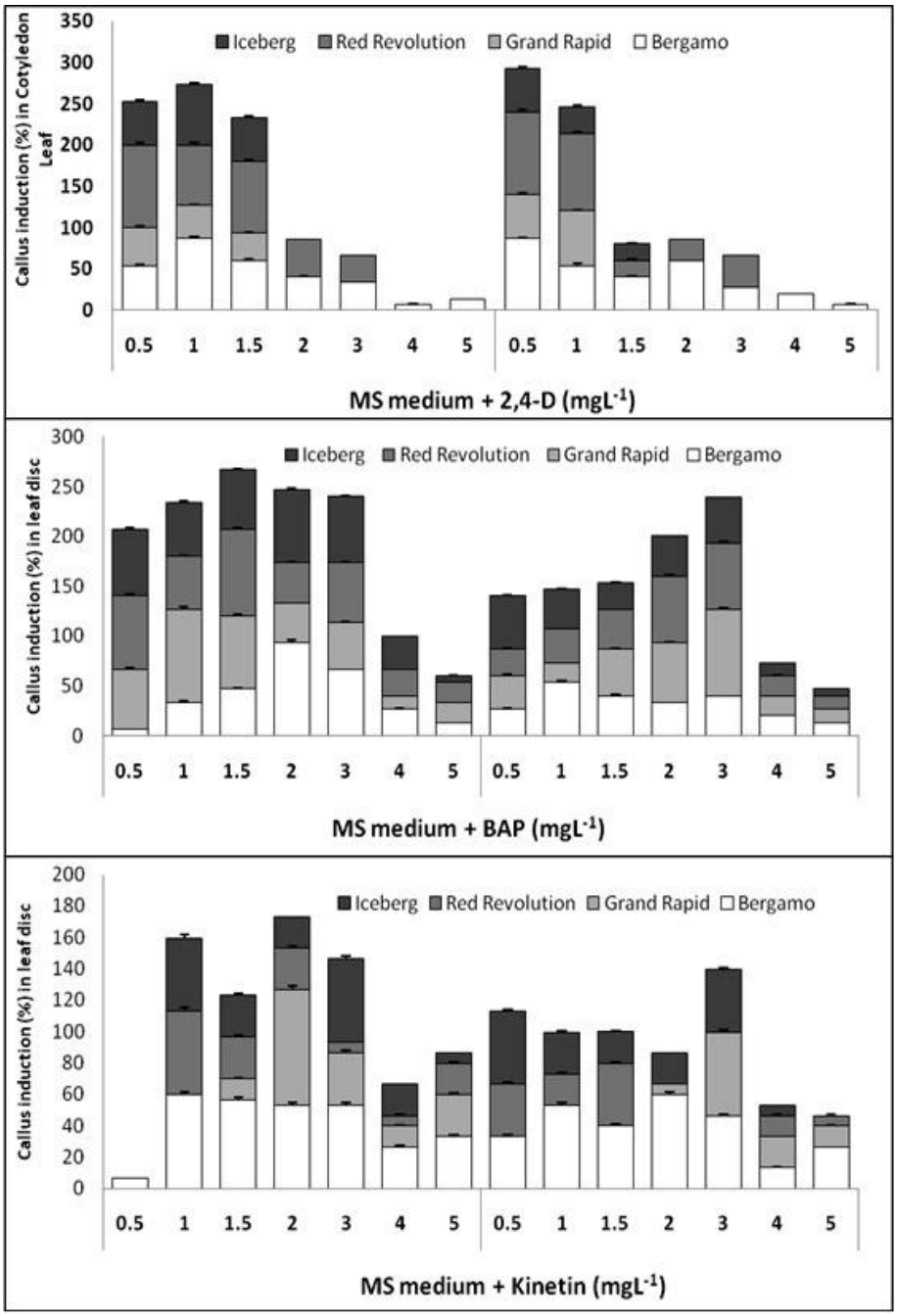

Fig. 3A-C. Photoperiod dependent genotypic response for callus induction (%) in Cot explants on MS medium containing 2,4-D (A), in leaf disc explants on MS medium containing BAP (B) and Kinetin (C) in lettuce under long days (LDs) and Dark (D) culture conditions. Data are means of 3 replicates plus standard error (SE) bars from two independent experiments with 20 explants per rep. Results are significantly different from each other at probability ($P<0.05$).

DISCUSSION

Leafy vegetables as important source of food and nutrition are widely used round the globe. Recalcitrant nature of spinach and lettuce cultivars signifies the establishment of efficient callogenesis protocol for future biotechnology applications in these crops. Like other environmental factors, photoperiodic alterations play a significant role in plant tissue behavior in response to up or down regulation of endogenous hormone production (Mengxi*et al.*, 2011; Neelakandan and Wang, 2012). The crosstalk of endogenous hormones

in the tissue and exogenous hormones applied in the growing media defines the tissue response (Gaj*et al.*,2005; Duclercq*et al.*,2011). The auxin 2,4-D has most frequently been used *in vitro* to initiate embryogenic calli in a variety of plant species like carrot, onion and cucumber (Lutharand Bohanec, 1999; Mashayekhi and Neumann, 2006; Usman *et al.*, 2011). The growing potential of meristemetic cells can be maintained in the medium containing 2,4-D followed by excessive callus formation. Auxins could be used alone or in combination with cytokinins for callogenesis and regeneration as reported using cotyledon explant of tomato on MS medium with 0.2 mgL^{-1} IAA by Ramiah and Rajappan (1996). However, no differences are reported in endogenous cytokinin levels in calli indicating its minor role in determining embryogenic capacity of carrot tissue cultures (Jimenez and Bangerth, 2001).

Different explants were explored for higher callus induction and proliferation in both vegetable crops. Explant age played a critical role in triggering callus induction in both spinach and lettuce cultivars and 15 days old Cot and Hyp explants produced more calli compared with 7 days old explants. In spinach, callus induction peaked at lower level of 2,4-D under LDs compared with D in Cot explant (Fig. 1A) with more callus induction in cv. Faisalabad local. In Hyp explant callus induction peaked at higher levels of 2,4-D under both LDs and D regimes inducing more calli in F1 Hybrid Philipino. These findings showed an explant type and genotype dependent callus induction behavior in spinach and suggest a probable higher endogenous auxin production in Cot explants compared with Hyp explants thus reducing the requirement of exogenous auxin supplement in the media. In contrast, callus induction in Lettuce cultivars peaked more in D at low 2,4-D levels compared with explants placed under LD conditions particularly in cv. Red Revolution (Fig. 3A). Our findings of LD requirements for callus induction in Spinach are supported by similar studies in seedling derived explants of ramie by Wang *et al.* (2008), *Dierma erectum* by Koetle *et al.* (2010) and a recent report in spinach by Milojevic *et al.* (2012). However, these reports are contrary to our findings in Lettuce that induced more calli under dark conditions. These differences could be attributed to differential crop and genotypic behavior *in vitro*. Differential requirement of photoperiod for *in vitro* response is also reported in *Huernia hystrix* by Amoo *et al.* (2009). Our findings of genotype and explant type dependent callus induction are supported by Milojevic *et al.* (2011) in spinach; Mohebodini *et al.*(2011) in lettuce and Usman *et al.* (2011) in cucumber however, the present study implicates different age of explants and callus induction on 2,4-D instead of NAA and BA.

Cytokinins BAP and KIN were separately used to induce callus induction in both Cot and leaf disc explants in Lettuce. LD conditions showed a direct impact on callus induction that peaked early at lower concentrations of BAP and KIN and gradually increased with rise in BAP level from 0.5-1.5 mgL^{-1} (Fig. 3B). In contrast, D conditions delayed callus induction at higher BAP and KIN levels ($3mgL^{-1}$) suggesting light stimulus may have enhanced endogenous cytokinin production in the explants thus reducing the exogenous hormonal requirement for callus induction in the media under LDs (Fig. 3C). Similar enhanced response for callus induction is reported by Milojevic *et al.* (2012) in spinach on GA_3 under LDs as endogenous GA_3 was reported to be higher in spinach under LDs by Metzger and Zeevart (1980). Our findings of callus induction on BAP and KIN are supported by similar response from Cot and Hyp explants in chilli on BAP and IAA by Ashrafuzzaman *et al*. (2009) and Cot, Hyp and leaf disc explants in sugar beet using BAP and 2,4-D under LDs by Gurel *et al*. (2001).

Conclusions: We demonstrate here the photoperiod dependent callogenesis behavior of spinach and lettuce cultivars. Callus induction response was strongly dependent upon explants age and type used in relation to exogenous application of 2,4-D and BAP in the media. These results may contribute to our better understanding in the development of proliferating calli from vegetative explants. These studies may be further useful for the transformation of important biomolecules in the edible leafy vegetables.

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