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Original Article

Somatic embryogenesis, embyogenic cell suspension from *Ocimum sanctum (L.)* leaf callus cultures and their rosmarinic acid accumulation

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ABSTRACT

The accumulation of rosmarinic acid (RA) in Ocimum sanctum (L.) somatic embryogenic callus and its cell suspension cultures was investigated. For callus induction, leaves, stems and inflorescence explants excised from field-grown plants were cultured on Murashige and Skoog (MS) medium containing 2,4-D (1 mg l^{-1}) combined with kinetin (0.1-0.5 mg l^{-1}), 2,4-D (0.5-2.5 mg l-1) individually, IAA (0.5-2.5 mg l^{-1}) individually, and NAA (0.5-2.5 mg l -l) individually. Due to better proliferation ability and high RA content of leaf callus, it was chosen to induce somatic embryogenesis by supplementing with different concentration and combination of BAP + NAA + kinetin. Among the different combination tested, BAP (1 mgl^{-1}) + NAA (1 mg l^{-1}) + kinetin (0.5 mg l -l) exhibited a maximum embryogenic potential (73%) and number of globular embryo (34.3) per callus. In order to study the RA production, the proliferation of embryogenic suspension culture was tested by transferring embyogenic callus at stationary growth phase from MS solid to liquid medium containing the same composition and hormonal concentration. After five subcultures, the suspensions were maintained continuously for six weeks without subculturing. Suspension cultures showed progressive callus growth and RA accumulation throughout the experimental period. The highest RA (82.1 mg l^{-1}) content and biomass growth (11.6 g l -1) was observed in the cultures supplemented with BAP (1 mg l^{-1}) + NAA (1 mg l^{-1}) + kinetin (0.5 mg l^{-1}). It was 40 fold higher than the leaf callus.

1. Introduction

Ocimum sanctum (family: Lamiaceae), commonly known as holy basil in English, is a fragrant bushy plant found in semi tropical and tropical parts of India. Different parts of this plant are traditionally used in the Ayurveda and Siddha systems of medicine for treating infections, skin diseases, hepatic disorders, cold, cough, malarial fever and as an antidote for snakebite [1]. Secondary metabolites are the compounds synthesized through phenyl propanoid pathway that have many roles including pathogen defense, color, odor etc[2]. Biotechnological techniques have been recently reported to significantly facilitate plant propagation and production of some important secondary metabolites from various plants. Some authors have varied the culture conditions in order to find a satisfactory equilibrium between the growth of cells and their production of these metabolites [3,4].

Of the various secondary metabolites reported, caffeic acid ester rosmarinic acid (RA; α-o-caffeoyl- 3,4-dihydroxyphenyllactic acid) is a natural antioxidant widespread in the families Lamiaceae and Boraginaceae [5,6,7]. RA has received particular attention because of its well known biological activities including antibacterial, antiviral, and anti-inflammatory properties[8]. Several studies have reported the production of this metabolite in callus and cell suspension cultures of Salvia officinalis[9], Anchusa *officinalis* [10] and *Coleus blumei* [11,12]. Recently we have reported the establishment of callus cultures from different explants of

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O. sanctum and its RA accumulation (Lukmanul Hakkim et al. 2007). In the present study, we report on the establishment of somatic embryogenesis from *O. sanctum* leaf callus cultures and its cell suspension by the influence of auxins and cytokinins and compared their RA productivity with that in leaf callus.

2.Materials And Methods

2.1.Plant material

The seeds of *O. sanctum* were purchased from the local market of Coimbatore. The plant was grown in the gardens of Bharathiar University. Green house plants were irrigated to pot capacity daily and maintained at day/night temperatures of 27–33 and 20–25ºC respectively. The plant was identified by the Botanical Survey of India, Southern circle, TNAU (Tamilnadu Agricultural University), Coimbatore. The specimen (Voucher specimen No. 231) was deposited in the department of Botany, Bharathiar University.

2.2.Callus induction

Leaves, stems, and inflorescence explants from 1-year-old *O. sanctum* plants were surface sterilized for 3–5 min in Tween 80 and for 3 min with 0.1% (w/v) $HgCl_2$ and then rinsed three times with sterilized distilled water. Sterilized explants were cut into 1 cm long pieces and cultured on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 3% sucrose (analytical grade) as a carbon source and 0.8% agar (Himedia Laboratories, Mumbai, India) for gelling, and growth regulators such as 0.5-2.5 mg 2,4-dichlorophenoxy acetic acid (2,4-D) l-l, 0.5-2.5 mg Naphthalene acetic acid (NAA) l -l, 0.5-2.5 mg Indole-3-acetic acid (IAA) l -1, and 2,4-D (1 mg l-1) combined with 0.1–0.5 mg of kinetin (KIN) l -1 were used. The cultures were maintained at 25 ± 2°C under alternative dark and light (1000–2000 lx) for 12-h photoperiods. Culture response of each explant for callus induction was recorded after 8 weeks. Data were expressed as mean SD of each three replicates.

2.3.Induction of somatic embryogenesis from leaf callus

Somatic embryogenesis was induced by transferring the leaf callus from MS solid medium supplemented with 2,4-D (1mg l^{-1}) + KIN (0.1 mg l^{-1}) to MS solid medium supplemented with benzyl amino purine (BAP) + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}), BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}), BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}) and BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}). All the cultures were maintained and stabilized for five weeks by subculturing at weekly intervals. Globular embryos per callus, embryogenic potential of callus by the hormonal supplementation as well as its RA content (by HPLC analysis as described below) at stationary phase were recorded. Data were expressed as mean ± SD of each three replicates.

2.4.Growth and RA accumulation in embryogenic suspension cultures

Embryogenic suspension cultures were established by transferring embryogenic calli, at stationary growth phase, grown and stabilized on MS solid medium supplemented with BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}), BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}), BAP + NAA + KIN (2 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}), BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 0.2 mg l^{-1}), BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}) and BAP + NAA + KIN (3 mg l^{-1} + 1 mg l^{-1} + 1 mg l^{-1}), to MS liquid medium containing the same composition and same hormonal supplementation. The suspension cultures of each hormonal supplementation started with six Erlenmeyer flasks of 250 ml capacity, each one, containing 50 ml of liquid MS medium and one friable callus tissue. After 15 d, a volume of 50 ml fresh medium was added to each flask and 15 d later the content of the six flasks from each combination of growth regulator treatment was combined and used for subculturing. After 30 d, in order to stabilize the cell suspension, subcultures were performed of the same hormonal combinations by transferring a volume of 10 m l suspension, to each Erlenmeyer flask of 250 ml capacity containing 100 ml of fresh medium for five times at weekly intervals. The cultures were maintained on a rotary shaker (120 rpm) at 25±2°C under 2000 lx illumination (12-h photoperiod).

In order to determine the effect of embryogenic suspension with growth regulator treatment on RA accumulation at the end of the fifth subculture, suspensions were maintained continuously for six weeks without subculturing. The embryogenic calli growth and RA accumulation in cell suspension were determined by harvesting the biomass periodically (once a week, throughout the six-week period) from the suspension by filtration. Data were expressed as mean SD of each three replicates.

2.5.Extraction and quantification of RA

The harvested biomass from both solid and liquid medium was dried, and the dry weight was determined [13]. RA content of dried biomass was determined as described earlier [14]. Data were expressed as mean ± SD of each three replicates.

2.6.HPLC analysis

A HPLC system comprising a vacuum degasser, quaternary pump, autosampler, thermostated column compartment, and photodiode array detector (PDA) was used for the quantification of RA. The column, phenomenex C_{18} 5 µm (250×4.6 mm), was maintained at 26°C. Different proportions of solvents such as acetonitrile/water/acetic acid (15:84:0.85) as eluant B and methanol as eluant A were used for the separation. The multiple gradient used for chromatographic separation consisted of different proportions of eluant A/B (50:50 for 1–5 min, 40:60 for 5–10 min, 30:70 for 10–15 min, 15:85 for 15–20 min). The flow rate was 1.0 ml min^{-1}, the sample injection volume was 50 µl, and the chromatogram monitored at 330 nm. The peak purity of the tested sample was determined by comparing its ultraviolet (UV) spectra to that of the reference standard. Quantification was made based on the corresponding peak area recorded by chromatopac c-R6A (Shimadzu). Standard RA (Sigma Aldrich, USA) was used for the preparation of calibration curves.

2.7.Statistical analysis

All data were expressed as mean ± SD of the number of experiments (n=3). The statistical significance was evaluated by one-way ANOVA, and significance of the individual comparisons was obtained by Duncan's multiple-range test (DMRT).

3.Results And Discussion

3.1.Culture response by the influence of growth regulators

Leaf, stem and inflorescence explants from field-grown young plant of *O. sanctum* were cultured on MS medium containing 2,4-D (0.5-2.5 mg l^{-1}), NAA (0.5-2.5 mg l^{-1}) and IAA (0.5-2.5 mg l^{-1}) individually for callus induction including 2,4-D (1 mg l^{-1}) + KIN (0.1-0.5 mg l^{-1}). Here we represented the response of growth regulators other than 2,4-D + KIN supplementation (Table 1). After 8 weeks of culture incubation indicated that medium containing 2,4-D (1.5 mg l^{-1}) showed maximum callus formation from stem (75.6%) whereas 2,4-D (1 mg l^{-1}) exhibited maximum callus induction from leaf (97%) and inflorescence (56.7%) respectively than the other growth regulators supplemented (Table 1). However, the obtained culture response is lower than the response obtained from leaf, stem and inflorescence callus cultures induced with 2,4-D + KIN supplementation [14]. Similarly, Dode et al. [15]have reported combination of auxin and cytokinin for callus induction. Our studies also accordance to Natasa bauer et al[16] and further revealed that 2,4-D and KIN combination is essential for friable green callus induction.

Table 1 :Effect of various concentrations of hormones on callus induction from *Ocimum sanctum* leaf, stem and inflorescence explants

Growth regulators ($mg1^{-1}$)	% of culture response		
	Leaf	Stem	Infloresence
0,5	60-1.4^{c}	50.6-2.1^{c}	25.5-1.6^{c}
1	97-2.3^{a}	70.4-2.1^{b}	56.7-1.7^{a}
1,5	85.2-1.8^{b}	75.6-2.0^{a}	45.4-1.2^{b}
2	50.2-1.6^{d}	46.5-1.4^{d}	23.5-1.1^{c}
2,5	35.2-1.5^{e}	30.6-1.2^{e}	15.3-1.1^{d}
NAA			
0,5	40.1-1.6^{d}	39.4-1.3^{c}	35.7-1.2^{d}
1	57-1.9^{a}	55-2.1^{a}	50.6-1.6^{a}
1,5	50.8-1.3^{b}	46.2-1.5^{b}	44.5-1.6^{b}
2	45.6-1.8^{c}	40.1-1.6^{c}	39.3-1.1^{c}
2,5	38.4-1.3^{e}	35.4-1.5^{d}	33.2-1.7^{d}
IAA			
0,5	45.6-1.8^{d}	44.5-1.6^{b}	40.5-1.4^{b}
1	60.7-2.4^{a}	58.9-1.5^{a}	52.6-1.7^{a}
1,5	55-1.9^{b}	47.5-1.2^{b}	49.3-1.5^{a}
2	49.3-1.1^{c}	38-1.7^{c}	37.4-1.3^{b}
2,5	35.6-1.2^{e}	32.4-1.5^{d}	28.9-1.1

Data were collected after 8 weeks of culture. "Values are given as mean ± SD for each three triplicates. Values not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

3.2.Somatic embryo formation and RA production in embryogenic cell suspension

Most studies on somatic embryo culture have focused on rapid propagation or fundamental work examining the differentiation and development of a wide variety of plants. In some cases, this technique has also been applied to the production of useful secondary metabolites such as furanochromone in *Ammi visnaga* [17], saponin in *Panax ginseng* [18], naphthoquinone in *Plumbago rosea* [19] and others [20]. Here we report for the first time RA accumulation in *O. sanctum* embryogenic callus cultures and its cell suspension. In this study, leaf callus obtained from MS medium supplemented with 2,4-D (1 mgl^{-1}) and KIN (0.1 mg l^{-1}) was used to investigate the influence of growth regulators on the induction of somatic embryogenesis based on its better proliferation potential and higher RA content (2.7 mg/g of dry weight: our published result). Induction of somatic embryogenesis from leaf callus was tried on MS medium with various concentrations of BAP + NAA + KIN. According to Godbole et al. [21], the 2,4-D and KIN combination was essential for initial culture establishment for callusing and subsequently, elimination of 2,4-D and a corresponding increase of BAP concentration induced somatic embryogenesis. Embryogenic potentialities of the *O. sanctum* leaf callus showed difference to some extend which depending on the growth regulators supplemented (Table 2).

Table 2 Effects of different concentration and combinations of BAP + NAA + KIN on the induction of somatic embryogenesis and rosmarinic acid content

Growth regulators ($mg1^{-1}$) BAP + NAA + KIN	% of somatic embryogenic potential of callus	Number of somatic embroyos /culture
1.0+1.0+0.2	61^{d}	25.2-1.1^{d}
1.0+1.0+0.5	73^{a}	34.3-1.3^{a}
1.0+1.0+1.0	58^{e}	21.4-1.6^{e}
2.0+1.0+0.2	62^{c}	26.6-1.2^{c}
2.0+1.0+0.5	65^{b}	27.1-1.1^{b}
2.0+1.0+1.0	66^{b}	28.1-1.4^{b}
3.0+1.0+0.2	63^{c}	26.1-1.2^{c}
3.0+1.0+0.5	43^{g}	15.6-1.1^{g}
3.0+1.0+1.0	51^{f}	17.3-1.1^{f}

Values are given as mean ± SD for each three triplicates. Values not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

In media, all the concentration of BAP + NAA + KIN combination showed that somatic embryogenesis started from the leaf callus after two weeks of subculturing. However, MS medium containing selected concentration of 1 mg l^{-1} of each BAP and NAA with additional KIN at 0.5 mg l^{-1} induced maximum embryogenesis than other concentration of hormonal combinations supplemented and embryogenic masses was observed as white yellow friable masses on the surface of calli (Fig. 1). It is concomitant with Junaid Aslam et al. [22] where Catharanthus roseus embryogenic mass was observed with similar morphology. Similarly, the same hormonal combination favored the maximum somatic embryogenesis in *Ocimum basilicum* leaf callus cultures [23]. The developed globular somatic embryos on leaf callus allow to continue growing on induction medium supplemented with same hormonal combinations for four weeks until heart and globular shaped forms appeared (Fig. 2). A preliminary chemical analysis of the matured *O. santum* somatic embryos revealed considerable RA accumulation in static cultures (data not shown). However, callus cultures are less suitable for studying the growth pattern and product formation than cell suspension cultures, due to limitations in homogenous mass and oxygen transfer to individual cells.

Fig. 1 Morphology of Calli biomass associated with embryogenesis. A) Initiation of embryogenesis in calli supplemented with BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.5 mg $^{-1}$) and arrow white indicates the site embryogenesis. B) Morphology of matured embryogenic calli after several subcultures (green color completely changed in to white yellowish).

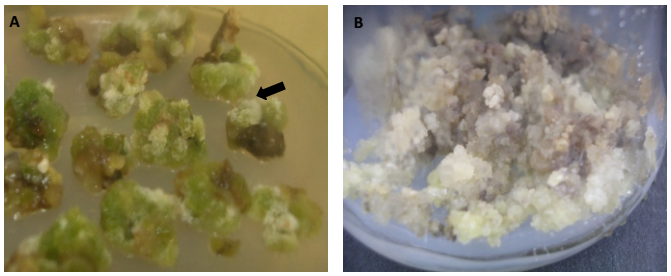
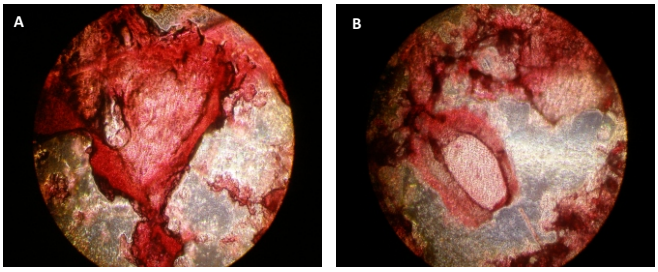


Fig. 2 Different stages of matured embryogenic callus cultured in solid medium supplemented with BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.5 mg $^{-1}$) after five subcultures A) Heart shape B) Globular stage.



Hence, the somatic embryo suspension culture was established by transferring the white yellowish friable masses of embryogenic calli at stationary phase to liquid MS medium containing the same composition and same hormonal supplementation. Further, the embryo suspensions were stabilized with the same hormonal supplementation by subculturing five times at weekly intervals. The cell suspension grown in the presence of growth regulators consisted of mainly embryogenic cells that were white cream colored (Fig. 3). Embryogenic callus growth was satisfactory in suspension compared to solid medium (data not shown). In order to reach a conclusion on the influence of embryogenic suspension with growth regulators on RA accumulation, the suspension cultures were observed six weeks continuously without subculturing after five subcultures. All callus lines exhibited progressively increased growth and RA accumulation during the exponential growth phase, which was observed throughout the experimental culture period (Figure 4 and 5). This result was in agreement with Kintzios et al. This is not surprising, since the secondary metabolism pathways are often restricted to an individual species or genus, which might be activated only during particular stages of growth and development [24].

Fig. 3 Morphology of suspension derived embryogenic biomass after six weeks of experimental period. The biomass was supplemented with BAP + NAA + KIN (1 mgl^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}).

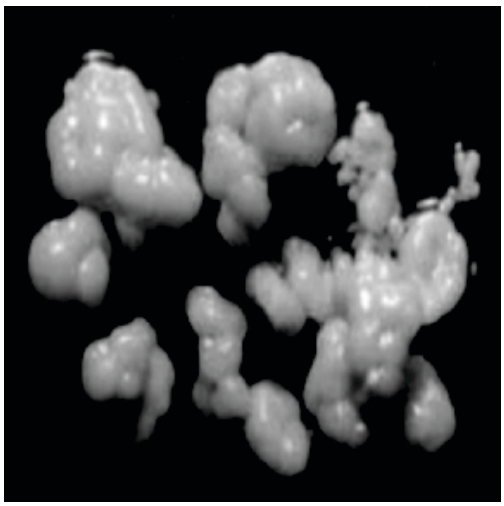


Fig. 4 Callus dry weight as affected by culture duration on MS liquid medium with the aid of different concentration and combination BAP + NAA + kinetin. Data were collected up to six weeks (once a week). Values are given as mean ± SD for each three triplicates. Values of combination of hormone concentration of each week not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

5. References

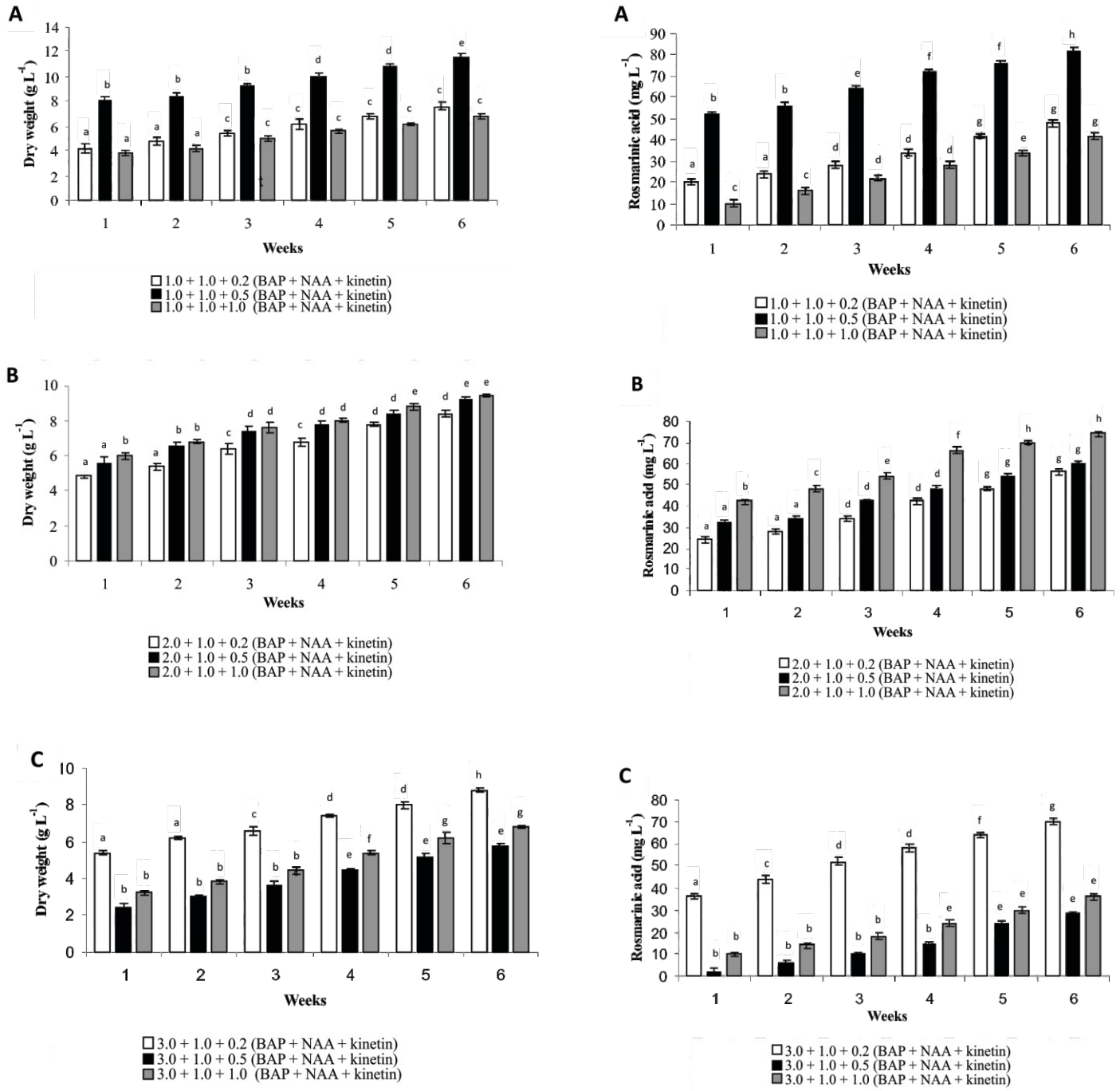


Fig. 5 Rosmarinic acid content as affected by culture duration on MS liquid medium with the aid of different combination BAP + NAA + kinetin. Data were collected up to six weeks (once a week). Values are given as mean ± SD for each three triplicates. Values of combination of hormone concentration of each week not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

In addition, the observed proliferation of embryogenic callus is suspension was somehow unexpected, since functional chloroplast are rarely present in cultured cells grown under normal laboratory condition, which leads to lack of photosynthetic activity and cell death [25]. The extend of chlorophyll synthesis in vitro, depends on the type of auxin and cytokinin used: BAP + NAA + KIN (which were used in the present study) is less detrimental

than other growth regulator combination. It is therefore possible that the investigated cultures retained part of their photosynthetic activity, which results efficient growth of embryogenic callus in suspension.

Further, in this study it was observed that RA is a growth associated compound, its accumulation being concomitant with increases in dry weight of biomass. Similarly, in *Hypericum calycinum*, the accumulation of adhyperforin is paralleled with cell growth and its formation increase with increased dry weight of biomass [26]. The maximal dry weight (11.6 g l^{-1}) was obtained from cultures supplemented with BAP + NAA + KIN (1 mg l^{-1} + 1 mg l^{-1} + 0.5 mg l^{-1}) at the sixth week, in parallel with a maximum RA (82.1 mg l^{-1}) content. The highest accumulation of RA in *O. sanctum* embryogenic suspension culture was 40 fold higher than the leaf callus.

In conclusion, the *O. sanctum* embryogenic suspension in the described medium conditions was efficient for RA accumulation. The observed pattern of RA accumulation in *O. sanctum* embryogenic suspension is novel and far more complex with production peaks coinciding during intense growth phase. Nevertheless, we further plan to elucidate RA production in O. sanctum embryogenic suspension by means of biotic and abiotic stress application.

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