The role of phenolic compounds in growth improvement of cultured tobacco cells after exposure to 2-D clinorotation

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Abstract

Previous studies have confirmed that the growth and development of plants are entirely dependent on the gravitational acceleration of the Earth. So far, most of the studies on the plant response to the Earth gravity have focused on the geotropism of root tip of higher plants. Under space and clinorotation conditions, however, plant growth and development are supposed to be modified. The aim of the present study was to evaluate the growth and metabolism of cultured tobacco (*Nicotiana tabacum* L. cv. Burley 21) cells after 1 week continuous treatment with 2-D clinorotation. In these cells, the sensing of gravity and response to it occur in single cells without the involvement of specialized organs or tissues. The contents of phenolic compounds and phytohormones were evaluated by HPLC. Growth parameters i.e., fresh and dry weight of 2-D clinorotation-exposed tobacco cells significantly increased and it was accompanied by a significant increase in the contents of Indole-3-acetic acid and brassinosteroids, compared to the control group. Exposure to clinorotation also increased the content of antioxidant phenolics but significantly decreased the content of lignin producing phenolic acids. Altogether, these metabolic alterations prevented membrane damage and maintained wall loosening and growth potential of tobacco cells.

Keywords: Brassinosteroid (BR); Clinorotation; Indole-3-acetic acid (IAA); phenolic compounds; tobacco cells

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Introduction

Regarding the regulatory role of geotropism in plant growth and development, it seems that the change of gravity from 1 g to microgravity conditions heavily affects the plant cell proliferation and growth (Matia et al., 2010). Microgravity provides a unique environment to assess plant physiological responses related to gravity.

Studying on the actively proliferating meristem root cells of *Arabidopsis thaliana*, Manzano and his coworkers (2013) observed a great disruption of meristematic competence under microgravity conditions. They also reported that both real microgravity and cllinotation

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decouple cell proliferation and cell growth in root meristem.

Previous studies showed that microgravity treatments caused specific effects on plants at the cellular level. Boucheron-Dubuisson et al. (2016) studied the effect of simulated microgravity on cellular functions of the root meristem of *Arabidopsis thaliana*. Using flow cytometric analysis, they observed changes in the proportion and duration of cell cycle phases. Moreover, the expression of the cyclin B1 gene, a marker of entry in mitosis, was decreased indicating that cell cycle regulation was altered by simulated microgravity.

Cultivating seedlings of rice on board the Space Shuttle STS-95 mission, Hoson et al. (2004) observed a spontaneous (automorphic) curvature of coleoptiles toward the caryopsis in the elongating region. There was a significant correlation between the angle of curvature and the difference in the cell wall extensibility of the convex and the concave sides. They attributed these phenomena to the lower ratio of high-molecular-mass polysaccharides in the hemicellulose fraction, but higher activity of (1-->3),(1-->4)-beta-glucanases in the cell wall of convex side.

Proteomics analysis of *Arabidopsis thaliana* callus after exposure to hyper gravity (8×*g*) indicated stress relief or adaptation to a new gravitational steady state under prolonged exposure (Barjaktarovic et al., 2009).

Proteomic profiles of Arabidopsis callus grown under microgravity on board the Chinese spacecraft SZ-8 however, showed that several proteins not previously known to be involved in the response to microgravity or gravitational stimuli, such as pathogenesis-related thaumatin-like protein, leucine-rich repeat extension-like protein, and temperature-induce lipocalin, were significantly up- or down-regulated by microgravity (Zhang et al., 2015).

Gene expression microarray analysis of Arabidopsis introduced induction of a novel transcription factor WRKY (WRKY46) with a unique spatial arrangement of *cis*-elements after exposure to simulated microgravity (Soh et al., 2012)

Changes in energy metabolism after exposure to simulated microgravity have been shown in cultured tobacco cells as a plant model cell line (Soleimani et al., 2019).

It has been suggested that a large number of abnormal physiological responses of plants to microgravity and clinorotation conditions are related to the elevated production of reactive oxygen species (ROS) in cells (Baranenko, 2001; Chen et al., 2015). Although ROS is produced during normal cell metabolism, their levels are enhanced by exposure to chemical and environmental stresses. ROS are powerful oxidants that significantly affect cell growth and can have deleterious effects on biological macromolecules such as DNA and proteins, as well as membrane lipids (Choudhury et al., 2017). Therefore, plants fight free radicals through their scavenging by antioxidant defense systems such as enzymatic antioxidants and various types of non-enzymatic antioxidants such as phenolic compounds (Ghanati et al., 2007; Choudhury et al., 2017).

Plant phenolic compounds are considered as parts of active defense responses and play roles in H_2O_2 reduction in the phenol-coupled ascorbate peroxidase reaction (Ducicet al., 2008).

Most of our knowledge about the effect of gravity on plants have been focused on the perception of gravity (gravitropism) through sedimentation of amyloplasts of specialized statocytes in roots according to the gravity vector. The effects of gravity and its alteration on the distribution of plant hormones and changes of cell wall architecture have also been studied in coleoptile and root cells of some plants (Kiss et al., 1999; Hoson et al., 2004). The present study was undertaken in order to deepen our understanding of the role of altered gravity vector (generated by 2-D clinostat) in the antioxidant system of cultured plant cells. Cultured tobacco cells have been used as an undifferentiated, embryonic, and vulnerable plant cell model system where the sensing and response to gravity simultaneously occur in single cells, without the involvement of specialized neighboring organs or tissues as it happens in seedlings (Kamal et al., 2015). It may help us to improve our general understanding about the intrinsic mechanism(s) of the responses of the living systems to microgravity conditions.

Material and Methods

Culture conditions and clinorotation treatment

A cell line of tobacco (*Nicotianatabacum* L. cv. Burley 21) was cultured in a modified LS medium without glycine and solidified with 0.8% agar. The medium was supplemented with 3% sucrose, 3 mg L^{-1} NAA, 3 mg L^{-1} IAA, 0.15 mg L^{-1} kinetin, 1.5 mg L^{-1} thiamin, 0.75 mg L^{-1} pyridoxine, and 0.75 mg L^{-1} nicotinic acid, pH 5.8 (Ghahremani et al., 2014). Cultures were grown in darkness under controlled conditions of the temperature (25 ± 2 °C) and the humidity (65% RH). The medium was renewed every 15 days.

A two-dimensional clinostat was used (Miyamoto et al., 1999). The device was a generous gift from the United Nations Office for Outer Space Affairs (Zero-Gravity Instrument Project: ZGIP; UNOOSA, Vienna). The device was sterilized by UV and ethanol (70%). Then, the tobacco calli were cultured at the center of solidified medium containing Petri dishes (the radiuses of the callus and the Petri dish were 1 cm and 8 cm, respectively). Samples were randomly divided into two groups. In the gravity group each Petri dish was placed exactly from its center on the axis of rotation of the device via double-sided tape. At each test, only one Petri dish was located on the clinostat. The control group was placed horizontally on the ground (g=1) and kept near the device under similar conditions in terms of the temperature and humidity (25±2 °C, 65% RH). Gravity samples were rotated in the order of 20 rpm for a period of one week, continuously. At the end of the time, the cells were harvested and their fresh and dry weights were measured. Aliquots of the cells were removed and frozen immediately in liquid nitrogen and stored at -80 °C for further biochemical analysis.

Microscopic analysis

The cells of both control and clinorotation-treated groups were fixed with FAA (formalin: acetic Acid: Alcohol; 2: 1:17) overnight. The cells were then washed thoroughly and stained by Feulgen method using Schiff's reagent, as a specific method for DNA and nucleus staining as follows: The cells were sequentially passed through cold HCl (1 N, 25 °C, 10 min), hot HCl (previously reached to 60° C, 10 min), and returned to cold HCl for 10 min. The cells were then stained with Schiff's reagent (1h) followed by de-staining in three separate sodium metabisulphite solution 10%, 10 min each (Jouni et al., 2012). The cells were observed under bright field microscope (Olympus BH2, Japan) equipped with a digital camera.

Biochemical analysis

Phenolic compounds were extracted and determined following the method described by Barreca et al. (2016). In brief, the cells were extracted in 3 mL of acidic methanol (MeOH: HOAc, 99:1, v: v) followed by centrifugation at 12,000 g for 15 min. The supernatant was collected and dried and re-dissolved in 700 µL methanol. The extract was filtered by a sterile syringe filter (0.22 µm). Aliquots (20 µL) of the filtrates were applied to HPLC system (Waters, e2695, USA). The system was equipped with an HPLC pump K1001 and a C18 column (Perfectsil Target ODS3, 5 µm, 250 × 4.6 mm, MZ-Analysentechnik, Mainz, Germany), and UV detector. Phenolic compounds were eluted at a flow rate of 1 mL min^{-1} with solvent A (dH_2O/HOAc, 97:3, v:v) and solvent B (MeOH) under the following gradient conditions: 0–3 min, 0% B; 3-9 min, 3% B; 9-24 min, 12% B; 24-30 min, 20% B; 30-33 min, 20% B; 33-43 min, 30% B; 43-63 min, 50% B; 63-66 min, 50% B; 66-76 min, 60% B; 76-81 min, 60% B; 81-86 min, 0% B and equilibrated 4 min for a total run time of 90 min. Injection volume was 50 µL, and the column was thermostated at 25 °C. UV spectra of phenolic compounds were recorded from 280 to 325 nm.

For determination of phytohormones the cells were extracted in 3 mL of absolute MeOH on an ice bath overnight. The extract was then concentrated via evaporation and filtered by a sterile syringe filter (0.22 µm). Aliquots (20 µL) of the filtrates were applied to HPLC system as mentioned above. Phytohormones were eluted at a flow rate of 1 mL min-1 with a gradient of MeOH: acidic water (deionized water containing 0.67% acetic acid, pH 3.0). The gradient was as follows: 0-0.6 min 60% B, 0.6-10 min 55% B, 10-20 min 85% B, 20-25 138 min 60% B. Indole-3-acetic acid (IAA),

Brassinosteroid (BR), and Abscisic acid (ABA) were detected at 220 nm, and were quantified by comparison of their retention times and peak area with genuine standards (Sigma) (Delavar et al., 2017).

Statistical Analysis

The experiment was conducted using a completely randomized design. All the experiments were repeated at least three times. In each repetition of clinorotation, the treatment and control group were picked from one batch. All of the data were expressed as the mean ± the standard deviation (SD). Statistical analysis was performed using the Student's T-test. The differences between the treatments were expressed as significant at P≤ 0.05.

Results

As shown in Fig. (I), exposure to 2-D clinorotation significantly increased the fresh and dry weights of tobacco cells, compared to the control groups (Fig. I. A-B). Staining of the cells with Schiff reagent showed two intact nucleoli in the nucleus of both control and clinorotation-exposed cells (Fig. II. A-C). The rate of cell division however, was remarkably higher in clinorotation-exposed cells than the control (Fig. II. C-F).

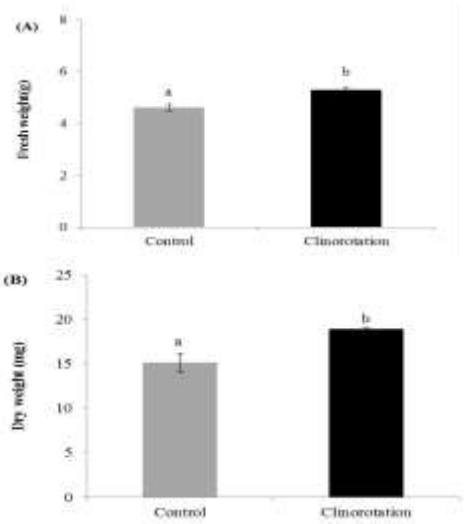

Fig. I. Stimulation of the growth of tobacco cells after exposure to clinorotation; data are presented as the means ± SD, n = 3. Bars with different letters represent significant differences at p ≤ 0.05, according to Student's T-test.

Table 1. The content of phenolic compounds and hormones characteristics of cultured tobacco cells treated with or without 2-D clinorotation; Ctrl, control; CR, clinorotation treatment

Phenolic acids	Control	Clinorotation
	(µg/g FW)	
Cinnamic acid	53.2±5.3[a]	80.4±5.8[b]
P-Coumaric acid	67.4±6.6[a]	90.1±5.4[b]
Gallic acid	25.9±2.5[a]	38.7±3.6[b]
Caffeic acid	8.2±0.6[a]	5.2±0.5[b]
Ferulic acid	11.4±0.1[a]	10.5±0.1[b]
Salicylic acid	43.8±3.1[a]	46.3±4.5[a]
Flavonoids	**(µg/g FW)**	
Catechin	10.8±0.2[a]	11.9±0.1[b]
Naringenin	9.7±0.5[a]	13.6±0.6[b]
Rutin	9.3±0.6[a]	16.4±1.3[b]
Syringin	11.8±0.4[a]	12.4±0.7[a]
Hormones	**(ng/g FW)**	
IAA	1560±40[a]	2330±70[b]
ABA	1.62±0.12[a]	1.59±0.09[a]
BR	1.17±0.03[a]	2.29±0.21[b]

Data are means ± SD, n = 3. Different letters indicate significant differences at p ≤ 0.05, according to Student's T-test.

The HPLC analysis was performed to identify and quantify phenolic metabolites in tobacco cells before and after exposure to clinorotation. Certain phenolic acids i.e., cinnamic, *p*-coumaric, ferulic, caffeic, salicylic, and gallic acid, and 4 flavonoids derivatives i.e., naringinine, rutin, syringing and catechin were quantified. As shown in Table 1, in comparison with the control conditions, the contents of cinnamic-, *p*-coumaric-, and gallic acid were significantly increased but the contents of caffeic- and ferulic acid decreased after clinorotation. The level of salicylic acid showed no change after treatment with clinostat, compared to the controls.

A remarkable increase was observed in polyphenolic compounds with high antioxidant activity i.e., naringinine, rutin, and catechin contents of clinorotion-exposed cell while their syringing level was identical to the control (Table 1).

Exposure of tobacco cells to clinorotation significantly increased contents of plant hormones i.e., BR and IAA, compared to the control cells (Table 1). No significant change, however, was

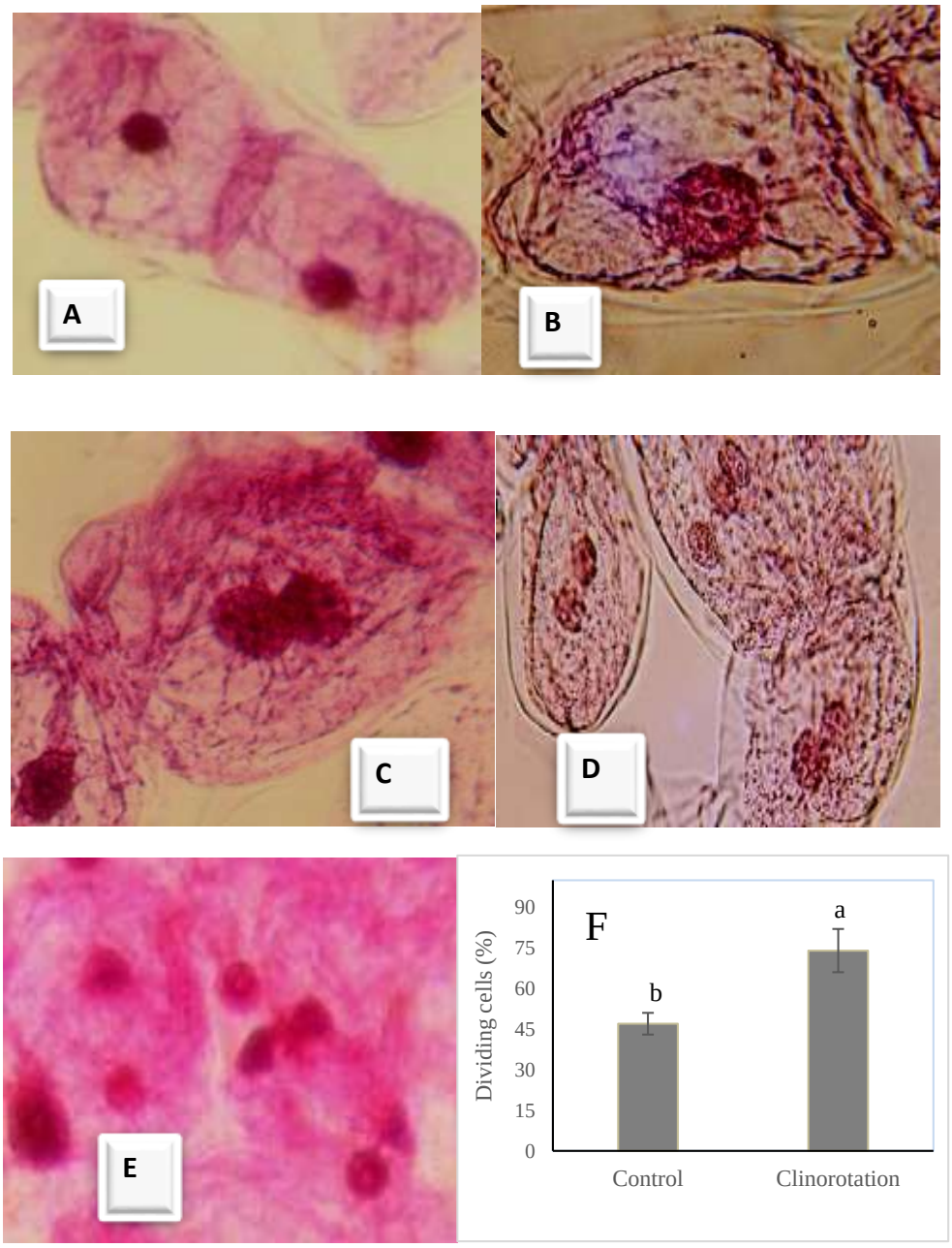

Fig. II. Nuclear phenotype, cell division, and growth characteristics of cultured tobacco cells treated with or without 2-D clinorotation; (A, B) control cells,(C-E) cells treated with 2-D clinorotation; note to the intact nucleoli in both control and treated cells (B,C); recently dividing cells are distinct with nuclei. Bars are equal to 40 µm in A, D, and E, and 30 µm in B, C.

observed in ABA content of the cells after clinorotation (Table 1).

Discussion

Earth gravity is a mechanical force that pulls objects towards the earth and alteration of the gravitational environment, i.e., changes of the forces acting on the cell is a significant environmental change. Therefore, it should be no surprise that single cells also react and adapt to gravity changes. Increased cell proliferation rate has been demonstrated under both real space flight and simulated microgravity (under

clinorotation) which in turn affects the growth and development of plants (Herranz and Medina, 2014; Hoson, 2014).

In the present study, improvement of the growth parameters of tobacco cells was observed with a significant increase in cells fresh and dry weight under changes in the gravity by using 2-D clinorotation. In root apical meristem of Arabidopsis, Boucheron-Dubuisson et al., (2016) observed morphological and morphometric nucleolar changes and reduction of ribosome biogenesis rate under simulated microgravity. However, in tobacco cells the nucleus and its two nucleoli were intact and identical to those of the control. Moreover clinorotaion provided the cells with a higher potential of division, resulting in the increased growth rate. This may clearly show the importance of the studies on the intrinsic response of individual plant cells to simulated microgravity independent of the other signals received from neighboring cells and surrounding tissues.

It has also been shown that the cells saved more energy under clinorotation than the normal conditions. Regarding the fact that under normal conditions the cells must expend energy to maintain their positional homeostasis against gravity, it is reasonable to suppose that under microgravity or clinorotation conditions, such energy may be saved for other processes, such as proliferation or biosynthesis of various primary and secondary metabolites (Soleimani et al., 2019).

Reactive oxygen species are normally produced as byproducts of cellular metabolism (Ghanati et al., 2007). In a recent mRNA-sequencing analysis on *Brassica rapa* grown in long-term spaceflight Sugimoto et al., (2014) observed up-regulation of 20 oxidative marker genes, including high expression of four hallmarks, and preferentially expressed genes associated with ROS-scavenging including thioredoxin, glutaredoxin, and alternative oxidase genes. This suggest that improvement of metabolism under microgravity and clinorotation conditions is potentially accompanied by the increase in ROS, which subsequently triggers the improvement of radical removing antioxidant system (enzymatic or non-enzymatic) of the cell. Phenolic compounds are secondary metabolites that are found naturally in all plants. Due to possessing frequent hydroxyl groups, these compounds are able to provide hydrogen to reactive oxygen and nitrogen species and terminate the radical production chain reaction (Valentao et al., 2002; Michalak, 2006).

It has been shown that phenolic compounds act as scavengers of superoxide anion ($O_2•^-$), hydroxyl (HO•-), peroxyl radicals (ROO•), and singlet oxygen ($^1O_2$). Moreover, acting as metal chelating agents, they potentially prevent the participation of transition metal ions (particularly iron) in reactions generating oxygen free radicals, of which the most dangerous is HO•, arising in biological systems mainly by a Fenton reaction (Aboul-Enein et al., 2007).

Exposure of tobacco cells to clinorotation significantly increased the contents of naringenin, catechin, and rutin, compared to the control. Therefore, it can be concluded that clinorotation reinforced radical scavenging performance of tobacco cells. Improved antioxidant enzymes activity under microgravity conditions has also been reported in different plants (Baranenko, 2001; Shagimardanova et al., 2010; Chen et al., 2015).

Biosynthesis of phenolic compounds is started with production of cinnamic-, *p*-coumaric-, and gallic acid followed by the hydroxylation and methylation of their derivatives to produce ferulic, caffeic, and synaptic acids which by conversion to phenolic alcohols provide lignin biosynthesis precursors and cell wall stiffening. Ferulic acid moieties also reduce the growth potential through cross linking with each other and with sugar residues of cell wall polysaccharides and

glycoproteins. Elongation and cell wall extensibility are inversely correlated with the increase in the content of ferulic and diferulic acid in the primary cell wall (De Oliveira et al., 2015). Significant decrease of ferulic and caffeic acid of tobacco cells after clinorotion treatment can maintain wall loosening and growth potential of tobacco cells. These results are consistent with reports of Stutte et al (2006) and Nedukha (1996).

Auxin and brassinosteroids are of plant hormones known to influence both cell division and cell elongation in various developmental contexts (Hardtke, 2007). It is also noteworthy

that auxin and brassinosteroids act in an interdependent and possibly synergistic manner. The involvement of auxin signaling pathway in response to microgravity has been reported (Aubry-Hivet et al., 2014). A comprehensive gene expression analysis of floral buds of Arabidopsis showed that alteration of gravity substantially changed the expression of genes involved in the biosynthesis of phytohormones ABA and auxin (Tamaoki et al., 2014). Coincident with these reports, clinorotion treatment of tobacco cells in the present study significantly increased auxin and brassinosteroid contents of the cells.

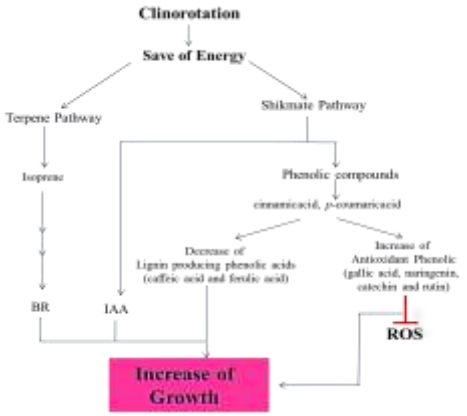

Fig. III. Schematic representation of interconnected network of phenolic compounds and hormones characteristics of cultured tobacco cells in response to clinorotation

Altogether, from the presented results it is likely that clinorotation provides the tobacco cells with improved growth via increase in non-enzymatic antioxidants, mainly phenolic compounds and also increase in auxin and brassinosteroids contents (Fig. III).

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