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Peroxidase activity, lignification and promotion of cell death in tobacco cells exposed to static magnetic field

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Abstract Bio-effects of static magnetic fields on cell growth and cell death have been investigated in suspension-cultured tobacco cells as undifferentiated, embryonic plant cell model. The cells in their logarithmic growth phase were exposed to static magnetic field with the magnitudes of 10 mT and 30 mT for 5 h/day. Exposure to static magnetic field ceased the growth and caused an increase in cell death of exposed tobacco cells compared to those cells which were not treated with the field. Promotion of cell death was accompanied by a harmonized increase in the activity of peroxidase and increase of lignifcation of cell walls.

Keywords Peroxidase activity · Lignification · Cell death · Static magnetic field · Tobacco cells

1 Introduction

Health effects due to exposure to static magnetic fields (SMFs) have become considerable public concern. There is no however, convincing scientific evidences that guarantee exposure to such magnetic fields is not hazardous for human health. In recent years several studies have suggested possible negative bio-effects of magnetic fields (Miyakoshi 2005; Zhang 2003). These fields can affect human health and lead to various types of cancer and genetic disorders. Although the exact mechanism(s) of these effects remain unknown, but one of the most recognized hypothesis is the radical pair's mechanism in which an external magnetic field, influences the kinetics of chemical reactions with radical pair intermediates. So it can be led to increase of the concentration of free radicals in the cells (Grissom et al. 1995). A potential link between magnetic fields and their effects on living organisms is the fact that MF causes an oxidative stress, that is, increase in the activity, concentration, and lifetime of free radicals (Zhang et al. 2003). Oxidative stress is a function of oxidative metabolites, free radicals, and reactive oxygen species (ROS) which are highly reactive by-products of normal metabolism as well as immune system (Dat et al. 2001). Oxidative stress can bring changes in gene expression, membrane structure and activity, cell growth, cell death; and thereby contribute to cancer and leukemia (Green et al. 1999).

There are only a few studies on the effects of magnetic fields on cell death. Apoptosis induction in human leukemia cells in vitro with high frequency magnetic fields has been reported; assessed by a DNA fragmentation method as a marker of apoptosis (Hisamitsu et al. 1997). Apoptosis is an active process fundamental to development and homeostasis of multi-cellular organisms. In another study effects of a low frequency magnetic field on DNA strand breaks in Rat brain cells have been investigated (Singh et al. 1998). Due to presence of these damages defensive reactions would be activated. It has been found that static magnetic field exposure influences the rate of apoptosis (spontaneous and induced), whose quality and quantity is dependent on cell type and on length of exposure (Luciana et al. 2005).

One of the typical examples of programmed cell death (PCD) in plant cells is the lignification of the tracheary element (vessels) differentiation. Lignification also occurs in plant cells under stress conditions, thereby limiting their growth (Morita et al. 2006). It is well accepted that in both

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cases the activity of various kinds of peroxidase are involved (Ghanati et al. 2005). Peroxidases have been suggested to be involved in various metabolic steps such as auxin catabolism (Normanly et al. 1995), the formation of isodi-Tyrosin bridges in the cross-linking of cell wall proteins (Schnabelrauch et al. 1996), the cross-linking of pectins by diferulic bridges (Amaya et al. 1999), and the oxidation of cinnamyl alcohols prior to their polymerization during lignin and suberin formation (Whetten et al. 1998).

Few studies however, have been conducted on a potential risk of plants exposure to the magnetic field and induction of oxidative burst, increase of peroxidase and lignification. Few available literatures, have particularly directed their attention to the effects of magnetic or electromagnetic fields on the germination of seeds and plant growth and development (Yano et al. 2004; Rakosy-Tican et al. 2005).

In the present study, we investigated the effect of magnetic field exposure on suspension-cultured tobacco cells as undifferentiated, embryonic, and vulnerable plant cell model. The goal was to determine a potential relationship between exposures to magnetic fields, cell growth and cell death (apoptosis, necrosis). It may help us to improve the general knowledge about mechanism(s) of the responses of the living systems to magnetic fields.

2 Materials and methods

2.1 Cell growth conditions

Suspension cultures established from calli of tobacco cells (Nicotiana tabacum L. cv. Burley 21) that had been maintained in our laboratory for 52 subcultures. Both calli and subsequent suspensions were grown in a modified Murashige and Skoog medium without glycine and containing 3% sucrose. The details of medium content as well as the cell growth conditions were presented elsewhere (Sahebjamei et al. 2007).

2.2 Magnetic field exposure

Exposure to MF was performed by a locally designed MF generator using a 220 V AC power supply equipped with variable transformer as well as a single-phase full-wave rectifier. The maximum power and passing current were 1 kW and 50 A DC, respectively. This system was designed to generate MF in range of 0.5 –30 mT. It consisted of two coils (each with 3,000 turns of 3 mm copper wire) on a U-shaped laminated iron core (to prevent eddy current losses). Using two vertical connectors, the arms of the U-shaped iron core were terminated in four circular iron plates covered with thin layer of nickel (each 23 mm thickness, 260 cm in diameter).

A water circulation system around the coils was employed to avoid the increase of the temperature. The temperature between the circular iron plates, where the samples were located, was measured and was almost the same as other parts of the room, for example the location site of the control cells, ±1°C.

The control cells were kept far enough from the MF producing apparatus, to avoid any potential exposure to the MF. Moreover, other electric appliances and laboratory facilities were not working; the control samples were therefore exposed only to the extremely low ambient MF (60 ± 5 μT), as the treatment groups were too.

Presence of any pulsation in the current from rectifier into the MF generating apparatus, was tested by an oscilloscope (40 MHz, model 8040, Leader, Japan) and a pulsation frequency of 50 Hz with a range of voltage variation about ±1 V, was shown. The presence of this pulsation frequency may be related to the shortcoming of the single-phase full-wave rectifier, which provides a ripple voltage around (5%). This small ripple voltage suggests that the generated MF can be considered highly homogeneous.

An electronic board was used to stabilize the system so that we always got a uniform MF. Calibration of the system as well as tests for the accuracy and uniformity of the MFs were performed by a teslameter (13610.93, PHYWE, Germany) with a probe type of Hall Sound. The accuracy of the system was ±0.1% for MF and the range of measurements was 3–30 mT.

The present study was undertaken to test the effects of 10 mT and 30 mT on plant cells. The cells in their exponential growth phase were continuously exposed to MF by magnitudes of 10 mT or 30 mT, for 5 days, from day 3 to 7 of subculture. After the period of treatment the cells were harvested and frozen in liquid N2 and kept at –80°C until used for biochemical analysis.

2.3 Extraction and assay of peroxidase

Peroxidase (PO) was extracted and determined in three fractions; the soluble (SPO), ionically (IPO) and covalently (CPO) bound fractions. The first one, using guaiacol as an electron donor, is involved in the stress response and the two latter, using syringaldazine as an electron donor, are supposed to be more related to the lignification and suberization of the cells (Pandolfini et al. 1992; Fukuda and Komamine 1982). Samples were homogenized in 50 m*M* Tris-maleate buffer (pH 6.0) and centrifuged at 1,000×g for 10 min at 2°C. The supernatant was re-centrifuged at 18,000 × g for 20 min at 2°C. This second supernatant was used to assay soluble PO. Pellets of the first and the second centrifugations were

pooled, incubated with 0.2 *M* $CaCl_2$ for 2 h at room temperature, and then centrifuged at 18,000 × g for 20 min at 2°C. The supernatant was used to measure the activity of IPO. The pellet was used directly for assay of CPO (Pandolfini et al. 1992). Activity of SPO fraction was assayed in 60 m*M* K-phosphate buffer (pH 6.1) containing 28 m*M* guaiacol and 5 m*M* H_2O_2. The increase in the absorbance was recorded at 470 nm. For IPO and CPO assay, the final reaction mixture (3 ml) contained 41.6 n*M* syringaldazine, 40 m*M* Tris-maleate buffer (pH 6.0) and 16 m*M* H_2O_2. Activity of IPO was expressed as the increase in absorbance at 530 nm per min per mg protein and activity of CPO was expressed as the increase in absorbance at 530 nm against cell wall dry weight.

2.4 Lignin content assay

In order to determine the lignin content of the samples, the cell walls were extracted (Ghanati et al. 2005). Proteins and lipids were omitted from cell homogenate and lignin content was measured by a modified acetyl bromide procedure in which measures the lignin concentration using light absorbance of the solvent using specific absorption coefficient (SAC) value 20.0 g^{-1} liter cm^{-1} in 280 nm wave-length (Iiyama and Wallis 1990).

2.5 Fluorescence microscopy

The cells were fixed with para-formaldehyde (4%) and phosphate buffer (pH = 7.4) for 10 min. A dye exclusion method was used to study cell death and morphological changes in the nucleus of the cells. In this case viable (with intact cell membrane) and dead cells (with damaged cell membrane) can be visualized by epifluorescence microscopy. In order to identify the ratio of viable and non-viable cells, specimens were stained with two DNA-florochroms: ethidium bromide and acridine orange. Fluorescence microscopy with differential uptake of fluorescent DNA binding dyes is a method of choice for its simplicity, rapidity and accuracy (Renvoitze et al. 1998).

Ethidium bromide do not penetrates the cell membrane in viable cells and stains only dead cells, but acridine orange penetrates the cell membrane and stains viable and non-viable cells. Apoptotic cells were identified according to their morphological features, such as nuclear fragmentation, nucleolus disappearance and chromatin condensation. Observations were made with an Olympus BH2 fluorescence microscope equipped with filter combinations WU (330–385-nm excitation and 420-nm barrier filters) and WBV (400- to 440-nm excitation and 475-nm barrier filters).

2.6 Statistical analysis

All of the experiments were carried out with at least five independent repetitions, each three samples, and all data were expressed as the mean values ± standard deviation (SD). Statistical analysis was performed using Student's t-test and the differences between treatments were expressed as significant at level of $P < 0.05$.

3 Results

The tobacco cells in suspension culture have different shapes such as round, cylindrical as well as ellipse. The dimensions of these cells were averaged on 30 cells and the results were presented in Table 1. These results show a statistically significant difference between unexposed tobacco cells and the cells which were exposed to 10 mT and 30 mT static magnetic fields. This process was repeated 10 times to avoid any possible statistical error. The average size of the treated cells with both 10 mT and 30 mT static magnetic fields was significantly decreased compared to those of the control cells.

The percentage of viability of tobacco cells (n = 30) before and after exposure to 10 mT and 30 mT static magnetic fields is presented in Fig. 1. When tobacco cells exposed to magnetic fields, there was a significant increase in the percentage of dead cells, compared to non-exposed cells. Figure 2 demonstrates morphological features of suspension cultured tobacco cells exposed to magnetic fields. Cleavage of nucleus and its vacuolization in tobacco cells exposed to 10 mT and 30 mT static magnetic fields in comparison with control cells is shown. Figure 3 indicates the activity of SPO, IPO and CPO, the different parts of peroxidase. The biochemical analyses were conducted at least three times, each three samples. As shown in this figure the activity of IPO was decreased but of SPO and CPO was significantly increased in SMF-exposed cells, compared to those of the control cells. Figure 4 shows the lignin content of cell wall in exposed cells compared with unexposed cells. These data come from conducting at least three times experiments, each with three samples. The last step in the synthesis of lignin and suberin has been proposed to be catalyzed by peroxidases.

Table 1 Dimension of suspension-cultured tobacco cells (n = 30) before and after treatment with SMF (Mean ± SD, n = 10)

Dimention (μm)	Magnitute of SMF (mT)		
	0	10	30
Length	49.2 ± 10.8	37.8 ± 10.8	37.2 ± 10.2
Width	41.8 ± 17.9	33.6 ± 8.3	29.3 ± 5.9

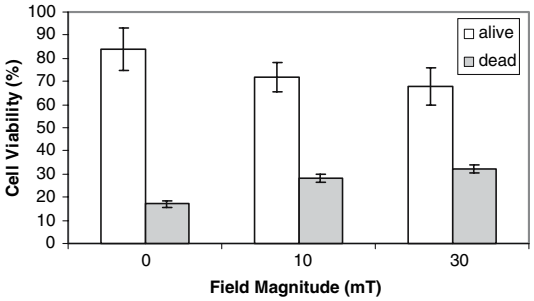

Fig. 1 Comparative histogram showing average results obtained for cell viability in treated cell at 10 mT and 30 mT compared with the results obtained for the control cells (n = 30)

4 Discussion

In previous research (Sahebjami et al. 2007), we showed that, magnetic fields can cause an inconsistency in the function of antioxidant enzymes in suspension-cultured tobacco cells; thereby lead to oxidative stress. Oxidative stress is a major cause of carcinogenesis, aging, and many other diseases (Ames 1983). Living cells and organisms are able to respond to a wide range of environmental stimuli and stresses, lead to intracellular and extracellular changes (Phillips et al. 1986) which can be classified as irreversible and reversible or structural and functional and changes to cells and their organelles. Oxidative stress may switch on many defensive processes in which affect cell growth and cell death as well as the activity of various enzymes.

It is unknown why cell growth would be suppressed by the presence of static magnetic fields (Sabo et al. 2001) and whether magnetic field adversely affect cell expansion or cell division. The data presented here, indicate that exposure to static magnetic fields affect cell growth by decreasing the size of the cells. On the other hand from decrease in the rate of viability in MF-exposed cells it can be concluded that SMF not only limited the expansion of tobacco cell, but also limited the rate of cell division. The mechanism by which magnetic fields could affect cell death is unknown. However, it is supposed that these effects may be mediated by increase of free radicals (Sahebjamei et al. 2007). Ishisaka et al. (2000) showed H_2O_2 -induced rapid fragmentation of DNA and a slow decrease in the viability of human cancer cell line HL-60. In the same cell line, Robison et al. (2002) presented data demonstrating that a decrease in the DNA repair may occur following oxidative burst induced by EMF. Although morphological features of programmed cell death and necrosis of plant cells, to some extent, differ from those in animal cells, in the present study some of these features, e.g., vacuolization of nucleus and disappearance of nucleoli were observed more in SMF-exposed cells, compared to the control cells. These features imply that exposure to SMF decrease survival of the cells and increase the appearance of signs of senescence and death.

A significant increase in SPO and CPO activity of SMF-treated cells was determined as well. The rate of the increases for SPO activity was near 61% and 25% in 10 mT

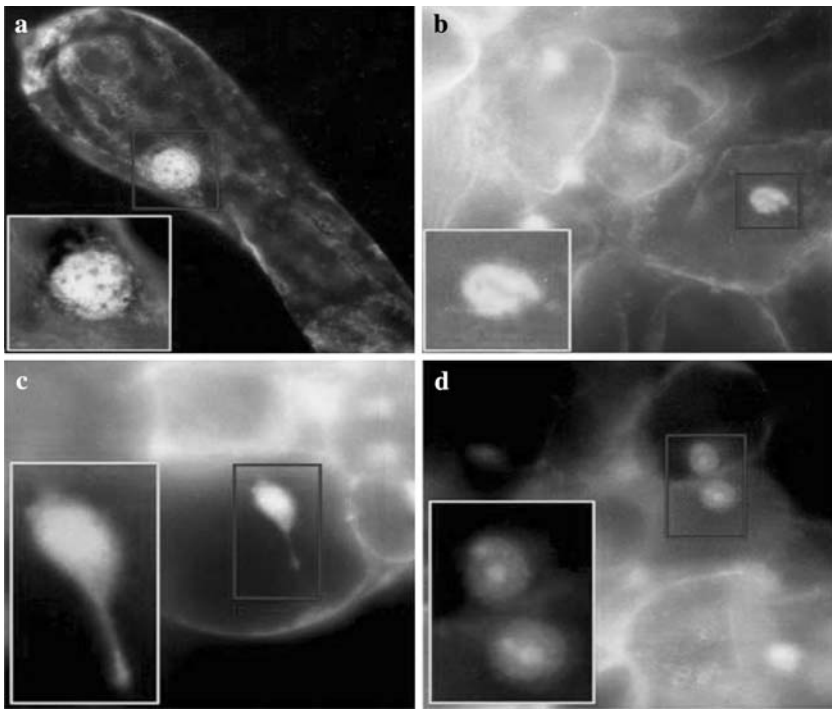

Fig. 2 Morphological features of suspension-cultured tobacco cells exposed to static magnetic fields. Cleavage of nucleus of tobacco cells exposed to 10 mT (**a**) and 30 mT magnetic fields (**b**, **c**), compared to the control cells with intact nuclei and clearly visible nucleoli (**d**). Cells were stained with acridine orange and observed under epifluorescence microscope. Magnifications of a, ×1,320 and of b, c, and d, ×660

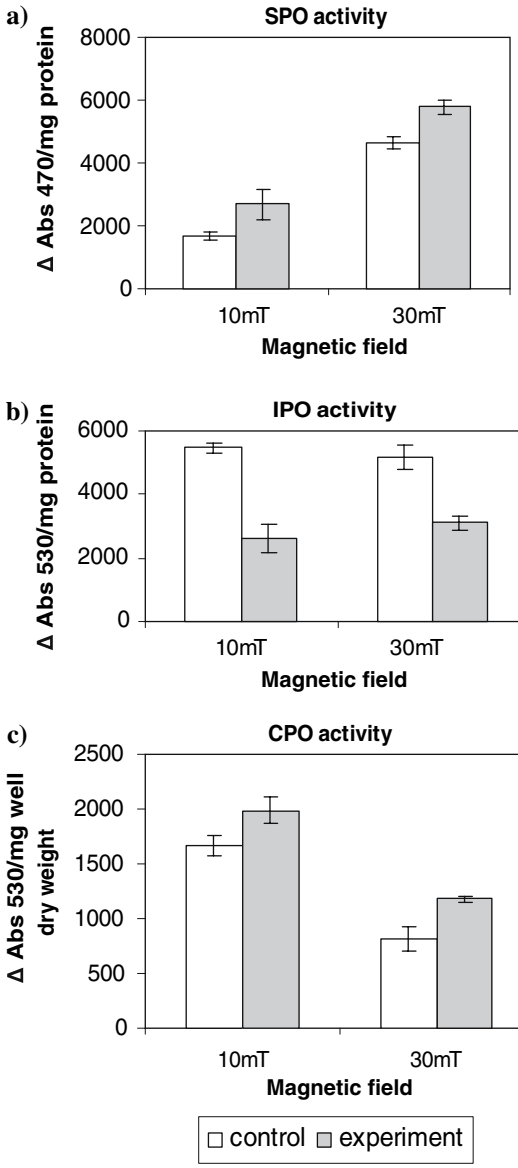

Fig. 3 Comparative histogram of the average effects of 10 mT and 30 mT static magnetic fields for the treated cells compared with the average results obtained for the control cells on peroxidase activity including, (**a**) the soluble peroxidase activity fraction, (**b**) the ionically peroxidase activity fraction, (**c**) covalently bound peroxidase activity fraction. Peroxidase activities were measured by recording the change in absorbance at specific λ (470 nm, 530 nm) against protein content as well as cell wall dry weight

and 30 mT static magnetic fields, respectively. The rate of the increases of the activity of CPO was near 19% and 46%, when the cells were treated with 10 mT and 30 mT SMF, respectively. This anionic type of PO has been supposed to be more related to the lignification of the cell walls (Ghanati et al. 2005; Pandolfini et al. 1992; Whetten et al. 1998). Coincident with this result, is the increase of lignin content of the cells exposed to static magnetic fields, compared to those of the control cells. It demonstrates the

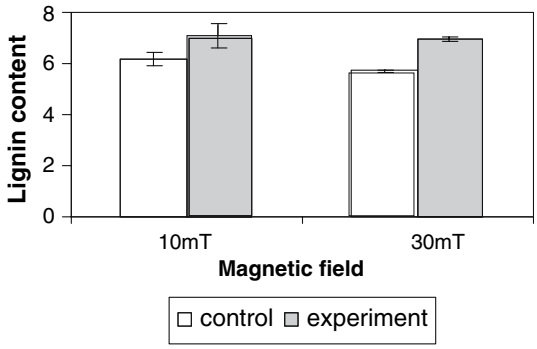

Fig. 4 Comparative histogram of the average effects of 10 mT and 30 mT static magnetic fields for the treated cells compared with the average results obtained for the control cells on lignin content of suspension-cultured tobacco cells

wall stiffening as a defensive task against oxidative stress and can possibly decrease the cellular growth and may be why the size of cells has been decreased.

Understanding the bio-effects of static magnetic fields and the mechanisms of their function would enhance our knowledge about possible relationships between magnetic fields and cancer, which is the goal of many related researches at present.

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