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High Rosmarinic Acid Content in Induced Mutants and in *in vitro* Elicited Sweet Basil (*Ocimum basilicum* L.) Callus

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Abstract: Seeds of Sweet basil (*Ocimum basilicum*) were exposed to different doses of gamma radiation and several mutants were selected upon morphological aberration. The spectrophotometric measurements showed that some mutants have 1.5 fold increase in the rosmarinic acid content comparing to the wild type. The *in vitro* shoot culture of mutants was established with fastest callus growth on MS media supplemented with 1 mg L^{-1} of 2,4-D and 0.25 mg L^{-1} of KIN. Addition of 5 g L^{-1} of yeast extract to the culture media led to increase the rosmarinic acid content with 3.4 fold further. The soluble and insoluble leaf protein fraction of mutants were screened on SDS PAGE. The protein patterns demonstrate that the soluble proteins have a more stable pattern than the insoluble proteins.

Key words: *Ocimum basilicum* L., callus, mutant, rosamarinic acid, protein electrophoresis, induction

INTRODUCTION

The genus *Ocimum*, Lamiaceae, collectively called basil, has long been acclaimed for its diversity. *Ocimum* comprises more than 30 species of herbs and shrubs from the tropical and subtropical regions of Asia, Africa and South America. Basil is a multipurpose medicinal herb. It is used as a kitchen herb and as ornamental in house gardens. Extracts of the plant are used in traditional medicines and have been shown to contain biologically active constituents that are anti angiogensis, antiviral, anti- inflammatory, antibacterial, powerful antioxidant, insecticidal, nematicidal, or fungi static (Huang and Zheng, 2006).

Basil is known to contain the antioxidant phenolic compounds mainly rosmarinic acid which is one of the most common caffeic acid esters occurring in Lamiaceae. Rosmarinic acid and some structurally related compounds have been proposed to be the active principals in crude preparation which display a range of physiological or pharmacological activities. Besides the antioxidative properties, rosemarinic acid has specific antimicrobial and antimicotic properties that are important for cosmeceuticals formulations (Petersen and Simmonds, 2003).

Cell and callus cultures have been used for studies of plant biochemistry because of their many advantages, for example, the ease with which environmental factors are controlled, exclusion of effects due to other tissues or microbial interactions and rapid growth.

Various *in vitro* cultures were established for production of rosmarinic acid from a number of plant plants which normally accumulate rosmarinic acid in the organized plant tissues, although only few of these cultures, namely *Coleus blumei* (Natasa *et al*., 2004), *Anchusa officinalis* (De-Eknamkul and Ellis, 1984), *Lithospermum erythrorhizon* (Mizukami *et al*., 1993), *Rosmarinus officinalis, Salvia officinalis and S. triloba* (Karam *et al*., 2003; Kintzios *et al*., 1999) and *Ocimum basilicum* (Kintzios *et al*., 2004) have been convincingly shown to continue producing rosmarinic acid *in vitro*. Typically, explants from phenolic-rich plant tissues such as these Lamiaceae plants darken when placed on the usual culture growth media, but the surviving tissue eventually produces a fast-growing, friable, light-colored callus. Unlike some secondary metabolite-producing cultures (Ellis, 1984), the production of rosmarinic acid appears to be constitutively expressed in these cell lines, some of which have been in continuous culture for 10 years with no reduction in metabolite yield (Li *et al*., 2005). Rosmarinic acid accumulation in callus or root cultures, in *in vitro* and *in vivo Ocimum* plants was studied. Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum* was reported (Peterosen and Simmonds, 2003).

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Gamma irradiation has been widely applied in medicine and biology in terms of biological effects induced by a counter intuitive switch over from low dose stimulation to high dose inhibition. Previous studies have shown that relatively low dose ionizing radiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Baek *et al*., 2005; Kim *et al*., 2005).

Until now Rosmarinic acid is produced commercially only with relatively low concentrations. This had limited its use to a very narrow number of applications. This breakthrough product line opens great opportunities for nutraceuticals and cosmeceuticals producers.

The main objective of this work was the increase of rosmarinic acid content in *Ocimum basilicum* plants by elucidation of Gamma mutants and subsequent induction of *in vitro* callus for higher production of rosmarinic acid.

MATERIALS AND METHODS

Irradiation experiment: Seeds of *Ocimum basilicum* were divided into nine groups and exposed to different gamma radiation doses; 0, 10, 20, 30, 40, 50, 60, 70 or 80 krad. The irradiation was performed using a Co^{60} source at a dose rate of 27.7 rad sec^{-1}. The seeds were sown in pots of 25 cm diameter filled with equal volumes of peat moss and sand. Five pots dose contained ten seeds were irrigated and fertilized twice a week. In the first two weeks, the pots were irrigated without Hoagland and by half Hoagland solution at the 3rd to 6th week and with full Hoagland at the rest period from the 7th to 10th week.

Technique for quantitative estimation of rosmarinic acid content in *Ocimum basilicum* L.: Estimation of rosmarinic acid content was carried out depending on spectrophotometry determination in both aerial parts and callus cultures. Rosmarinic acid content was extracted and measured according to Kintzios *et al.* (2004).

SDS-PAGE of leaf proteins: Five gram of fresh weight leaves of sixty days old plants were frozen in liquid nitrogen and manually grounded. The soluble protein fraction was extracted with 5 mL of extraction buffer (20 mM Hepes, pH 8.0; 4 mM magnesium acetate; 0.4 mM EGTA; 2 mM DTT; 40% glycerol). After centrifugation 10 min at 15000 rpm the sediment tissue was extracted with SDS buffer to extract the insoluble proteins (1% SDS; 60 mM tris, pH 6.8; 2 mM EDTA; 10% glycerol). The soluble protein content was determined according to the methods described by Bradford (1979). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the banding patterns of the mutants. Protein fractionation was performed on vertical slab (16.5×18.5 cm, Hoefer E600, Amersham Phrmacia Biotech) according to the method of Laemmli (1970), as modified by Studier (1973).

***In vitro Ocimum basilicum* (plantlets and callus) as a source of rosmarinic acid:** Shoot tips of *Ocimum basilicum* were washed thoroughly with running tap water for about 30 min and immersed in 30% commercial chlorox solution (a disinfectant containing 1.5% sodium hypochlorite) with 2-3 drops of tween 80 (as wetting agent). After shaking for 10 min, the shoot tips were washed three times with sterile distilled water. The cultivation of shoot tips were carried out using (Murashige and Skoog, 1962) without plant growth regulators. The incubation conditions were 25±2°C at 16 h day^{-1} photoperiod (2000-2500 lux).

For callus induction, the shoot tips were cultured on twelve different combinations of MS basal medium supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} meso-inositol, in all media and 0.25 or 0.50 or 1.00 or 2.00 mg L^{-1} of 2,4 dichlorophenoxyacetic acid (2,4-D) in combinations with different kinetin (KIN) concentrations (0.25 or 0.50 or 1.00 mg L^{-1}). the recording of callus fresh mass was starting from the second week of the culturing period until the 8th week. The efficiency of each medium variant for callus formation was determined according the Growth value equation (Szoke *et al*., 1970).

Where:

G_e = Mass (mg) of callus at the end of each incubation period,

G_{start} = The starting mass (mg) of the callus.

Elicitors dosage response: Callus was subcultured using 3 g of fresh callus in 40 mL of MS basal medium supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} meso-inositol, 2.00 mg L^{-1} 2, 4-D, 2.00 mg L^{-1} NAA, 10 mg L^{-1} ascorbic acid and various concentrations of yeast extract (0.00, 1.00, 2.00, 3.00, 4.00 and 5.00 g L^{-1}) as an elicitor.

Statistical analysis: The experiments were achieved according to the completely randomized block design. Statistical analysis for comparing among means was applied using the Least Significant Difference (LSD) as described by (Snedecor and Cochran, 1982).

RESULTS

The wild type seeds of *Ocimum basilicum* were exposed to five different doses of gamma radiation 10, 20, 30, 40 and 50 Krad (Table 1). Cultivation of irradiated seeds demonstrated that the dose of 50 Krad completely inhibited the seed viability. Mutants with apparent

Table 1: Rosmarinic acid concentration and total soluble protein content of leaf samples

Treatments	Total soluble protein content (µg µL⁻¹)	RA content mg L⁻¹ DW
Control	10.5	11.75
10 k Rad	7.1	10.41
	8.3	12.25
	5.4	5.29
	8.3	13.81
	8.0	8.98
20 k Rad	7.1	12.07
	7.5	11.26
	8.6	8.78
	9.2	12.14
	6.3	12.08
30 k Rad	7.6	18.17
	7.8	5.93
	8.8	13.06
	9.9	14.79
	6.9	9.03
40 k Rad	11.5	13.32
	14.4	11.96
	12.8	15.63
	12.2	12.55
	7.1	8.93

morphological differences was selected for further investigations. Most of the morphological aberrations included malformations of leaf shape, mosaic chlorophyll, abnormal internodes size and plant sterility Fig. 2.

The protein fractionation of the mutants was used as a tool for mutant genotypes characterization. Each protein preparation was divided to soluble and insoluble fraction. The determination of protein content in soluble protein samples showed the increase in protein content as the radiation dose increased (Table 1). The general aspect of insoluble protein banding pattern was the presence of two major bands one at ~45 kDa and the second at ~10 kDa. The high conservation of 10 kDa band in most samples might reflect its vital function. The odd exception of this pattern was that of sample number 17 which was exposed to 40 Krad (Fig. 1), where the 10 kDa was disappeared and new bands with higher molecular weight (duplication of 10 kDa) was evolved. This abnormal protein pattern was associated with fertile and

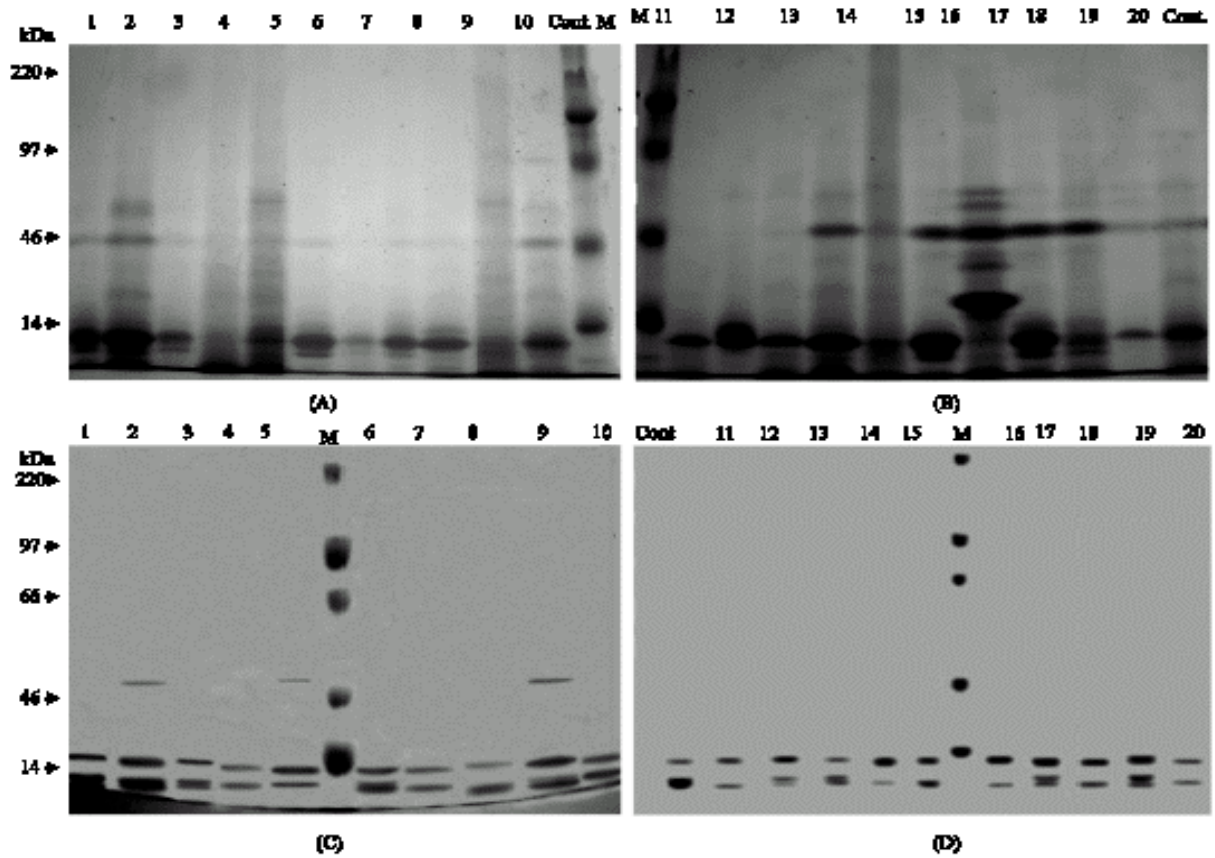

Fig. 1: SDS-PAGE for insoluble (A and B) and for soluble (C and D) protein of *Ocimum basilicum* mutants leaf tissues. M: standard protein molecular weight marker. Lane 1, 2, 3, 4 and 5: plants treated with 10 Krad. Lanes 6, 7, 8, 9 and 10: plants treated with 20 Krad. Lane 11,12,13,14 and 15: Plants treated with 30 Krad. Lanes 16, 17, 18,19 and 20: Plants treated with 40 Krad. Control: Plant untreated with Gamma-irradiation

Table 2: Effect of Different Elicitors on rosmarinic acid content of *Ocimum basilicum* after 8 weeks from culture

Treatments	Media component	Rosmarinic acid content (mg g^{-1})
Control	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid	6.70
1	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid + 1 g L^{-1} YE	7.69
2	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid + 2 g L^{-1} YE	7.73
3	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid + 3 g L^{-1} YE	16.72
4	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid + 4 g L^{-1} YE	20.01
5	MS + 2 mg L^{-1} NAA + 1 mg L^{-1} KIN + 100 m g L^{-1} ascorbic acid + 5 g L^{-1} YE	23.02
LSD at 0.05		1.88

vigorous morphological structure. This may interpreted as an effect of duplication of 10 kDa protein. The increase in band intensities was associated with samples of high radiation dose (30 and 40 krad). The soluble protein fractions were exclusively restricted at low molecular weight range (= 14 kDa).

Mutant screening for high rosmarinic acid content: The selected mutant plants were tested for rosmarinc acid content. Data in (Table 1) showed clearly that gamma radiation doses (10 to 40 krad) induced variation both in rosmarinic acid and total soluble protein content of *Ocimum basilicum*. Leaf sample screening for rosmrinic acid content showed a high increase in rosmarinic acid content for the samples number 11 and 21 (30 and 40 Krad mutants, respectively). The 30 Krad mutant has 1.5 fold increase comparing to the control sample while the 40 Krad mutant has 1.4 fold increase. The morphological study of those mutants showed some degree of plant infertility and weak vegetative growth. In addition, the 30 Krad mutant exhibited malformation for the leaf biogenesis, as the leaf structure is asymmetric. On the other hand, sample screening revealed the presence of other mutant with low rosmarinic acid content comparing to the control (0.7 fold of the control). This mutant is associated with leaf mosaic appearance.

Effect of plant growth regulators on *in vitro* callus and rosmarinic acid elicitation: The mutant plants with high rosmarinic acid content which had a problem with fertility were evolved in *in vitro* tissue culture as a favorable way for plant propagation and manipulation. Data (Fig. 2) clearly showed the effect of plant growth regulators and incubation periods on *in vitro* growth of *Ocimum basilicum* callus which was originated from stem, leaves and shoot tip. The growth parameters of callus culture indicated that the best medium for callus growth of *Ocimum basilicum* was MS basal medium supplemented with 0.25 mg L^{-1} kin and 1.00 mg L^{-1} 2,4 D which recorded 1422 mg culture callus fresh weight. The last media was followed by the nutrient medium containing 0.25 mg L^{-1} 2,4 D and 1.00 mg L^{-1} kin which recorded 1124 mg culture callus fresh weight.

The most interested potentiality of *in vitro* culture is the induction of callus to produce important secondary metabolites. Different elicitors were tried to increase the rosmarinic acid contents in the callus tissue over 6 week incubation period (Table 2). In callus culture, the rosmarinic acid content was increased proportionally with amount of yeast extract added to the culture media. More than three fold increase has been obtained when nutrient medium was supplemented with 5 g L^{-1} yeast extract was used.

DISCUSSION

Using of irradiation strategy is a well known method to get abnormal increasing amounts of already presented compounds in plant. The mutation effect on the metabolic pathways could be explained in term of one or more of the following four criteria, amplification of regular pathways (Chappie *et al*., 1992), blocking of competing pathways (Varin *et al*., 1992) and minimizing response cascades (Bailey, 1991; Nessler, 1994).

Selection of mutant plants for further biochemical analysis was carried out on the base of abnormal morphological criteria. The protein SDS PAGE has been used for further characterization of the selected mutants. The insoluble protein fractions showed a conserved band at ~10 kDa, the stability of this protein over the different protein patterns of the selected mutants reflect its vital function. However, the only deviation pattern was sample 17 where other band with duplicated molecular weight substituted the 10 kDa band. This mutant has a vigorous vegetative and high fertility ratio. The positive effect of molecular weight duplication may related to the supposed effect of this protein. The band intensity was increased in the case of mutant plants comparing to the control and this has been found in the results of Nayeem *et al.* (1999) on wheat; Farag (1999) on sunflower; Aparna-Das *et al.* (1999) on potato. However, the soluble protein fraction showed protein pattern at low molecular weight range. The major soluble protein in the cytoplasm and nucleus (histones) have molecular weight =14 kDa.

Biochemical determination of rosmarinic acid in mutants elucidate 1.5 fold increase in rosmarinic acid content in two mutants. These mutants have a small vegetative growth comparing to the control and are sharing one developmental character of plant sterility. The 30 Krad mutant has an interested malformation during the leaf development that high as shown in Fig. 2. The increase in rosmarinic acid content might be concerned

Fig. 2: Mutant with high rosmarinic acid content, B; Mutant with low rosmarinic acid content

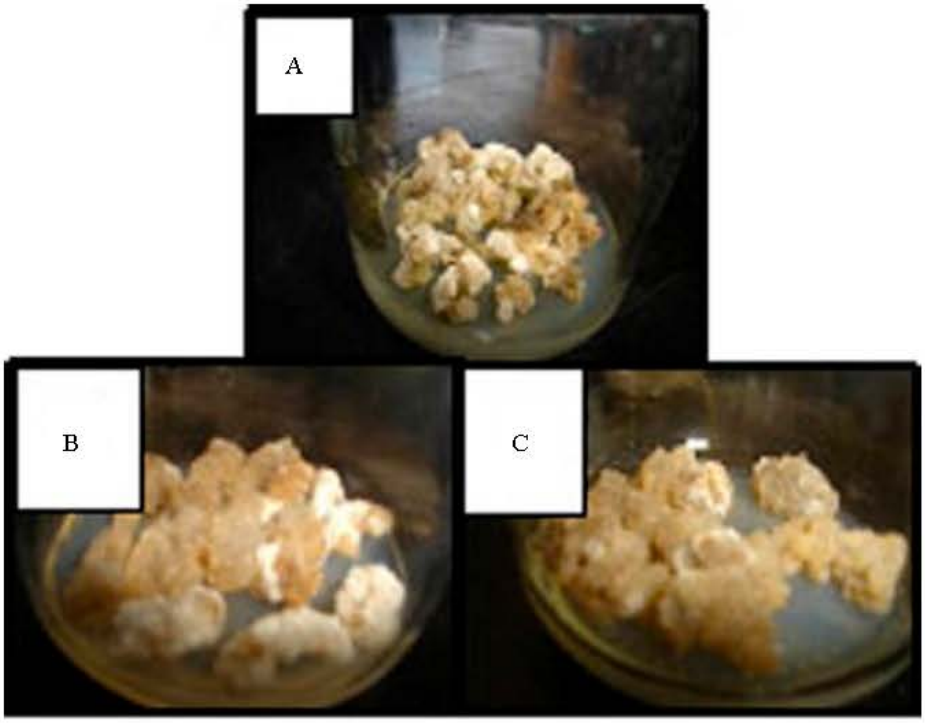

Fig. 3: Callus development from different *Ocimum basilicum* mutant explants, A: shoot tip, B: stem and C: leaf

with blocking of developmental pathway of plant maturity and reproduction or not and could be the subject for more investigations. The mutant screening has also reported the low rosmarinic acid content mutants with 0.7 ratio comparing to the control. In this case, the plant showed chlorophyll mosaicism in leaves Fig. 2.

In vitro culture of plant mutants with high rosmarinic acid content has been tested for further induction of the

compound. The stem culture on MS medium supplemented with 0.25 mg L^{-1} KIN +1.00 mg L^{-1} 2,4-D for 8 weeks gave the highest callus fresh weight. Several authors mentioned that the production of RA is initiated at the end of the exponential phase and increases exponentially during the linear phase of the growth cycle. This pattern indicates that the biosynthesis of RA is maximally induced during part of the growth cycle in which the rate of cell division has slowed down (Fig. 3). The progressive increase in the difference between the rate of RA synthesis and the rate of biomass accumulation ultimately results in a marked increase in the percent content (dry weight) during that particular period. Thus, the production/growth patterns of RA-producing cell cultures are actually similar to those of many plant cell and microbial cultures in batch culture system where the production is inversely related to cell division. This is the so called trophophase-iodophase development described by Bu'Lock (1975) and Luckner *et al.* (1977). According to this concept, idiophase is characterized by metabolic disturbances which lead to the accumulation of precursors or intermediates. Secondary metabolism, then, becomes active, either as a result of specific enzymeinduction or by the action of preformed enzymes on the accumulated substances. The nature of the hypothetical metabolic disturbances is likely to be complex and for this reason empirical approaches have generally been adopted in the studies of those cultural factors which influence the accumulation of secondary metabolites in plant cell culture (Mantell and Smith 1983).

This concept is in line with De-Eknamkul and Ellis (1985) who mentioned that the yield of both biomass and RA responded synchronously to changes in macronutrient and phytohormone concentration. Furthermore, both parameters attained their highest values at the same optimal concentrations of various macronutrients and hormones. This implied that the cell growth and RA formation are coupled to one another. These observations raised the question of how such coupling could occur when cell growth and product formation took place predominantly in different phases of the growth cycle. One possible explanation for this was that the final total content of RA might be a function of the length of time over which the initial growth rate declines. The more rapid the growth rate attained in log phase cells, the longer the subsequent period over which the growth rate would slow down and the higher the RA content ultimately obtained. The prerequisite of an initial high growth rate for a high RA content would, in turn, mean that an initial nutrient supply that promotes cell growth would also eventually promote RA formation. In this fashion, cell growth and RA formation would respond in a coupled fashion to nutrient supply changes.

The induction of rosmarinic acid content of *Ocimum basilicum* mutants after 6 weeks from culture was increased more than three fold when nutrient medium supplemented with 5 g L^{-1} yeast extract. In this respect, Purohit (1999) mentioned that two new techniques which have been developed : Hairy root culture and Elicitation of product accumulation. The simplicity of the procedures and outstanding results made the techniques most useful.

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