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Original Article

Effects of light and differentiation on gingerol and zingiberene production in callus culture of *Zingiber officinale* Rosc.

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Abstract

Ginger (*Zingiber officinale* Rosc.) is a herbaceous, rhizomatous, perennial plant, which its rhizomes are used as an important spices and medicine all over the world. The aromatic principles include zingiberene, while the pungent principles are known as gingeroles. Callus culture of the plants can produce the same compounds exist on their parent plants. The aim of this work was to evaluate the effect of light and differentiation on gingerol and zingiberene production in callus cultures of *Z. officinale*. A sterile *in vitro* plant was prepared by sterilization and subculture of buds, excised aseptically and inoculated into sterile culture jars containing Murashige and Skoog's (MS) medium and were incubated at 25 ± 2 °C under a 16/8 h light/dark cycle. Then different parts of the sterile plant were inoculated in MS medium supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid and kinetin. The jars were separated into two groups, one incubated in dark and the other one in light environment, permanently. Production of metabolites was evaluated by TLC. Some of the metabolites were produced only in presence of light. No gingerol and zingiberene was detected on TLC plates of the dedifferentiated callus grown in light or dark environment. It seems that the production of gingerol and zingiberene in callus culture of *Z. officinale* is correlated with some sort of differentiation.

Keywords: *Zingiber officinale*; Callus; Light; Differentiation

INTRODUCTION

Zingiber officinale is an aromatic plant used in spice industry and herbal medicines. The plant is widely cultivated in most tropical countries; India, Bangladesh, Taiwan, Jamaica, Nigeria, Indonesia, Ceylon, Sierraleone, Australia, China and Japan. India is the world largest producer of ginger (1,2).

The most important compounds, responsible for ginger's therapeutic activities, are divided into non-volatile and volatile compounds. The non-volatile fraction consists of an oleoresin. Elements responsible for gingers spicy flavor have been identified as the gingerols (3). The composition of the volatile fraction consists mainly of sesquiterpene derivatives, responsible for the aroma. Such compounds include (-)-zingiberene, (+)-curcumene, (-)-β-sesquiphelandrene and β-bisabolene. The monoterpene derivatives are also a part of this essential oil (4).

Studies suggest that ginger may be safe and effective for nausea and vomiting during pregnancy when used at recommended doses for short periods of time. It has been shown that [6]-gingerol is endowed with strong antioxidant activity both *in vivo* and *in vitro*, in addition to strong anti-inflammatory and anti-apoptotic actions (5).

The essential oil of rhizomes of *Z. officinale* plantlets in various media contains the same constituents as those of the original rhizomes. However, there are considerable differences. These differences seem to be caused by differences in the basal medium composition

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Table 1. Different hormone treatments for callus establishment

Treatmet No.	MS + GHRs
1	2,4-D* (1 mg/l)
2	2,4-D (1.9 mg/l)
3	2,4-D (3.5 mg/l)
4	Kinetin (0.2 mg/l)
5	2,4-D (1 mg/l)+ Kinetin (0.2 mg/l)
6	2,4-D (1 mg/l)+ Kinetin (0.2 mg/l)+ coconut milk

*D: dichlorophenoxy acetic acid

and the level of growth regulators added to the medium. A number of chemical and physical factors that could influence secondary metabolism in plant cell cultures have been found, including light, temperature, pH of medium, aeration, cell density, etc (6).

The main aim of this piece of work was to evaluate the effect of light and differentiation on secondary metabolites production in callus cultures of *Z. officinale*.

MATERIALS AND METHODS

In order to get a sterile plant, fresh buds, separated from the rhizoma of *Z. officinale* Roscoe imported from China, were used.

Culture jars were incubated at 25 ± 2 °C under a 16/8 h light/dark (L/D) cycle. Two months after establishment, the plant started to grow and differentiated. Then different parts of this sterile *in vitro* plant were used as explants for making callus culture. According to some studies, a mixture of auxins and cytokinins is a good pattern for a callus culture media (7,8). Therefore, in the present study, the Murashige and Skoog's (MS) medium used was supplemented with combinations of growth regulators presented in Table 1. The cultures were inoculated with different parts of the sterile plants (grown on MS medium) at 25 ± 2 C and then divided into two groups with different light regimen. One group was incubated under a continuous dark cycle in incubator and the second group was incubated under a 16/8 h L/D cycle.

For detection of differentiation process, thin sections from different stages of growth namely dedifferentiated, semi-differentiated and differentiated were prepared and stained with methyl green. First, it was seen under stereoscope to make the best segments, and then was seen under the microscope.

TLC analysis

Fresh callus samples (>1 g) of *Z. officinale*, grown in both dark and light environment and also the control sample (rhizome) were cut into small pieces and extracted under pressure with dichloromethane (5 ml) as the solvent, at room temperature. Then they were shaked for 20 min, filtered, concentrated under nitrogen gas to give at least 1 ml of the extract, dehydrated by anhydrous sodium sulfate and chromatographed on a pre-coated TLC silica gel plate (Silica gel G 060 F_{254} plates).

The TLC plates were put inside the developing tank for the solvent to run up. n-hexane: diethyl ether (40:60) used as developing solvent system. Then the plates were taken out before the solvent front reach to the top. The dried plates were then visualized by heating at 110 °C after spraying the reagent. The reagent used was sulfuric acid + vanillin. The individual zones were detected and identified. Comparison was made with the relevant literature (9).

RESULTS

Effect of light on callus growth

There was a little difference in callus growth at the same temperature in light environment and continuous dark. Some of the callus turned brown and died within a few months. The growing friable callus was white to cream in color, and composed of large translucent callus. Some of them gave rise to hairy root like structures that were composed of very tightly packed cells.

Secondary metabolites production

Several spots with different R_f values were detected in TLC analysis. Among them gingerol and zingiberene were identical. As presented in Table 2, no gingerol and zingiberene was detected on TLC plates of the dedifferentiated callus grown in light or dark environment. However, after spraying the reagent, one band in dark treated sample and two bands in light treated samples were

Table 2. Detected spots at different stages of growth (sulphuric acid+vanillin)

Treatment	Stage of growth			
	Dedifferentiated	**Semi-differentiated**	**Differentiated**	**Color**
Dark	R_f~ 0.35	R_f~ 0.35	-	Light violet
Light	-	gingerol	gingerol	Violet
	R_f~ 0.3	R_f~ 0.3	R_f~ 0.3	Light violet
	R_f~ 0.35	R_f~ 0.35	R_f~ 0.35	Light violet
	-	zingiberene	zingiberene	Dark purple

detected. In light treated semi-differentiated callus, a light violet weak spot was seen which was equal to the standard spot at $R_f \sim 0.2$ which refers to gingerol. Also one dark purple spot was seen at $R_f \sim 0.9$ which refers to zingiberene.

DISCUSSION

Expression of secondary metabolism and morphological differentiation

Differentiation was seen on callus mass on medium NO 5. However, differentiation progressed up to the second stage (semi-differentiated) in dark treated samples and to the third stage (differentiated) in light treated samples. This showed that light is a necessary factor for a complete differentiation.

According to Table 2, gingerol and zingiberene were produced along the differentiation process. None of them were detected in samples obtained from stage 1 but, both were presented in samples obtained from stages 2 and 3. It seems that gingerol and zingiberene production is a differentiation dependent process. There is a positive relationship between product accumulation and morphological differentiation, although unorganized callus tissue also seems to possess the necessary biochemical machinery to produce and accumulate some phenolic pungent principles. It is known that the complex metabolic changes which accompany the differentiation of cells in plants initiate biosynthetic pathways which result in the accumulation of new compounds.

Secondary products are normally found in highly differentiated parts of plants and constitute an important facet of differentiation. Secondary products may be formed as a consequence of differentiation (10).

It has been reported that the yield of desired products was very low or sometimes not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells (11). Matkowski discussed that a close correlation existed between the expression of secondary metabolites and morphological and cytological differentiation (12). It is not known whether these two processes are genetically and/or physiologically linked. The development of a certain level of differentiation is considered to be important in the successful production of phytochemicals by cell cultures. There are many examples in the literature demonstrating the relationship between differentiation and secondary metabolic accumulation. Hiraoka and Tabata reported that alkaloid levels increased in *Datura Innoxia* as shoots, stems and roots that were differentiated (13). In *Digitalis purpurea* culture, Hagimori et al. showed stimulation of digitalis cardenolides production by organ redifferentiation in callus tissues (14). A similar phenomenon was also found in rotenone formation using *Origanum vulgar* shoot culture (15) Kutchen et al. investigated the correlation between the stage of morphological differentiation and producing ability of the alkaloids using *P. bracteatum* and found a green callus with differentiated epidermis orvascular bundles which produced the alkaloids. At limited degree of tissue differentiation the cell contained codeine as the main alkaloid while the level of morphine increased as differentiation progressed (16).

Our results support this idea that secondary metabolites production (gingerol and zingiberene) depends on differentiation.

Light and expression of secondary metabolites

It is well known that light is a physical factor which can affect the metabolite production. As presented in Table 2 only in light

treated samples gingerol and zingiberene were obviously detected on TLC plates of semi differentiated and differentiated callus. So, it can be concluded that light can stimulate such secondary metabolite production in *Z. officinale* callus culture. An additional TLC spot with $R_f \sim 0.3$ was detected only on light treated callus samples of *Z. Officinale*. This spot was detected in all stages of growth. This unknown compound seems to be a light dependant but differentiation indepen-dent metabolite.

The interesting point to note is that although gingerol and zingiberene were seen on light treated samples, a spot with $R_f \sim 0.35$ was seen on both dark and light treated samples in all stages of growth (Tabel 2). So, it might be concluded that this metabolite can be consider-ed as a precursor metabolite and its production does not depend on differentiation or light treatment.

There are many reports that shows the light is an important physical factor which influence the growth and formation of primary and secondary metabolites (17-24). Similar to our results, stimulatory effect of light on the formation of secondary metabolites have been reported in *Panax ginseng* (17), *Peteroselinum hortense* (18), *Perilla frutiescnens* (19) *Artimisia annua* (20), *Rudbeckia hirata* (21) and *Hypericum perfaratum* (22).

Also, it has been shown that accumulation of secondary metabolites and induction of antioxidant enzymes was strongly stimulated by light in cell culture of in *Eleutherococcus enticosus* (23). Illumination was found to affect the composition of sesquiterpenes in callus culture of *Marticaria chamomile*. Also, exclusion of light in callus cultures of *Citrus limon* prompted the accumulation of mono-terpenes (24).

However, it has been reported that light is not always a stimulating factor for secondary metabolite production. For example light had an inhibitory effect on the accumulation of secondary metabolites such as nicotine and shikonin (25). It can be concluded that light can stimulate such secondary metabolite production in *Z. officinale* callus culture.

In general, it seems that the production of gingerol and zingiberene in cultured cells of *Z. officinale* is correlated with some sort of differentiation. So, this finding may show that secondary metabolits production in callus culture of *Z. officinale* is mostly related to chemical and structural nature of the compounds.

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