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Effect of Light and Reductones on Differentiation of *Pleurotus ostreatus*

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Vegetative mycelia of *Pleurotus ostreatus* were differentiated into primordia and subsequently into fruit bodies in synthetic sucrose-asparagine medium when exposed to light at low temperature. During photomorphogenesis, L-ascorbic acid-like substances called reductones were produced. L-Ascorbic acid, D-erythroascorbic acid, 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid were accumulated initially in the illuminated mycelia before the initiation of fruiting. The content of glycosides of erythroascorbic acid and their methylated compounds increased again in the primordia and the fruit bodies. Exogenous L-ascorbic acid induced the formation of primordia from the mycelia in the dark in a dose-dependent manner. Thus, this suggests that these reductones might play a role in mediating the light stimulus in photomorphogenesis.

***Keywords*:** reductone, light, ascorbate oxidase, photomorphogenesis, *Pleurotus ostreatus*

Pleurotus ostreatus is an edible basidiomycete. The haploid uninucleate basidiospore germinates and produces a homokaryotic mycelium. Two compatible strains fuse to form a dikaryon. After continuous growth in the substrate, dikaryotic mycelia develop into primordia and subsequently into fruit bodies bearing the hymenium.

Vegetative mycelia of *P. ostreatus* produce fruit bodies only when certain genetic conditions are fulfilled. In most cases, mycelia capable of producing normal fruit bodies result from the plasmogamy of two genetically compatible haploid mycelia. Moreover, the development of fruit bodies from dikaryotic vegetative mycelia that are genetically able to fruit is influenced by various environmental factors such as light (Eger *et al*., 1974), temperature (Zadražil, 1974), humidity, carbon dioxide (Zadražil, 1975), and carbon/nitrogen nutrient sources. These factors act together to induce the formation of primordia and subsequent fruit bodies from the vegetative mycelia. These factors might exert their effects by altering biochemical and developmental pathways. However, the precise mode of action is still unknown.

Light has long been known to be required for fruit body formation in some species of basidiomycetes (Eger *et al*., 1974; Manachère, 1980). It is likely that the light reception system and signal transduction pathways are involved in this developmental process, but little is known about the reception system and the transduction pathways. Light might exert its effect by stimulating the formation of biochemical substances that participate in the reactions leading to fruit body formation. However, little information is available regarding such substances.

L-Ascorbic acid is a representative reductone found in plants and animals (Szent-Györgyi, 1928). L-Ascorbic acid, D-erythroascorbic acid and an additional four reductones such as 5-O-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-O-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-O-(α-D-glucopyranosyl)-D-erythroascorbic acid and 5-methyl-5-O-(α-D-xylopyranosyl)-D-erythroascorbic acid have been identified in the fruit body of the oyster mushroom *P. ostreatus* and other basidiomycetes (Okamura, 1994; Lee *et al*., 2009). The enzymes involved in the ascorbate system were isolated from the vegetative mycelia of *P. ostreatus* and characterized. L-Gulonolactone oxidase containing flavin as its prosthetic group was responsible for the synthesis of L-ascorbic acid (Kwon, 1991). Ascorbate oxidase containing heme as a prosthetic group, catalyzed the oxidation of L-ascorbic acid to dehydroascorbate and hydrogen peroxide was produced during the oxidation (Kim *et al*., 1996; Lee *et al*., 2009). Ascorbyl radical reductase was responsible for recovery of L-ascorbic acid from the ascorbyl free radical (Yu, 1999). The extreme sensitivity of the ascorbate system to physiological changes implies that the ascorbate system may play a biochemical role in photomorphogenesis. Thus, the metabolism of L-ascorbic acid may provide a clue for elucidating the molecular mechanism of photomorphogenesis in *P. ostreatus.*

In this report, we describe reproducible culture conditions for light-induced fruit body formation from the vegetative mycelia, the contents of L-ascorbic acid-like reductones in the process of photomorphogenesis and their correlation with the onset of differentiation in a basidiomycete, *P. ostreatus*.

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Materials and Methods

Organism

The vegetative mycelia of different strains of the oyster mushroom, *Pleurotus ostreatus*, NFFA 2, NFFA 2M1, NFFA 2M2, NFFA 4001, and NFFA 4501 were obtained from the National Federation of Forestry Association in Korea and the KFCC 11635 strain was obtained from the Korean Federation of Culture Collection. The cultures were maintained on a yeast malt extract (YM) agar slant at 4℃ and sub-cultured every 4 weeks.

Chemicals

L-Ascorbic acid and methanol were purchased from Merck. D-Erythroascorbic acid was synthesized according to the method described previously (Kang, 1985; Liang, 1990). Dithiothreitol, 2,6-dichlorophenol indophenol and trifluoroacetic acid were purchased from the Sigma Chemical Company. Other reagents used were of analytical grade and were obtained from various commercial sources.

Medium

Synthetic sucrose-asparagine (SA) medium and complex yeast malt extract (YM) medium were used to determine a suitable nutrient condition for fruit body formation as described previously (Lee *et al*., 2009). The YM medium was composed of 3 g of malt extract, 3 g of yeast extract, 5 g of bacto-peptone, and 10 g of glucose per liter of distilled water. The SA medium contained the following constituents per liter of deionized water (Prillinger and Molitoris, 1979): 20 g of sucrose, 0.88 g of asparagine, 2 g of $NH_4H_2PO_4$, 1 g of L-valine, 0.224 g of $K_2HPO_4{\cdot}3H_2O$, 0.803 g of KH_2PO_4, 0.99 g of $MgSO_4{\cdot}7H_2O$, 0.02 g of $CaCl_2$, 5 ml of trace element solution, 5 ml of vitamin-nucleotide solution. The trace element solution contained 0.89 g of $ZnSO_4{\cdot}7H_2O$, 0.765 g of $MnSO_4{\cdot}H_2O$, 0.73 g of Fe(III)-citrate, 0.20 g of $CuSO_4{\cdot}5H_2O$ and distilled water to 1,000 ml. The vitamin-nucleotide solution contained 1.60 g of adenosine, 0.02 g of thiamine-HCl and distilled water to 1,000 ml. To prepare solid agar medium, 15 g of agar powder was added to 1 L of medium solution.

Culture conditions for determining the effects of nutrition, temperature, and light

Dikaryotic mycelia were grown in Petri dishes (10×10 cm) containing 25 ml of the YM or SA medium. Each dish was inoculated with an agar plug removed from stock cultures using a sterile cork borer. The inoculum plug was inverted and placed in the center of the culture dish. The mycelia were grown in continuous darkness at 25±2℃ for 8 days and then further incubated under the following conditions for more than 15 days: 1) continuous darkness, 25±2℃; 2) continuous darkness, 8±2℃; 3) continuous light, 25±2℃; 4) continuous light, 8±2℃; 5) alternate light and dark periods (8 light h and 16 dark h per day), 25±2℃; 6) alternate light and dark periods (8 light h and 16 dark h per day), 8±2℃. Illumination was provided by fluorescent lights (F40LW/RS/EW-II, Philips). The light intensity was 1300-1700 Lux. The relative humidity was 55±5%. All experiments were prepared with at least five replicates.

Measurement of reductones during photomorphogenesis

Vegetative mycelia were harvested from the Petri dishes every other day after the beginning of the secondary incubation. The mycelia were rinsed with double deionized water and were weighed. Primordia and fruit bodies were harvested from the Petri-dishes and were weighed. Harvesting was done under white light for light cultures or under red light for dark cultures. Mycelia, primordia, and fruit bodies were immersed in 1.5 vol of 95% methanol. Samples were frozen, thawed, and centrifuged. The supernatant was used for the determination of reductones as described previously (Lee *et al.*, 2009). The supernatant was loaded onto two tandem-linked ODS hypersil columns (Hewlett Packard, 100 mm×4.6 mm), and chromatographed on a Waters 510 HPLC equipped with a 460 electrochemical detector. The eluent was 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The potential of the detector was set at 0.80 V vs. an Ag/AgCl reference electrode. A stock solution of L-ascorbic acid was prepared at a concentration of 100 ng/ml with double deionized water just prior to the analysis. After serial dilution, aliquots (10 μl) of each standard solution were analyzed and a standard curve was constructed by plotting peak area vs. amount of L-ascorbic acid.

Assay of ascorbate oxidase during photomorphogenesis

The activity of ascorbate oxidase in the extracts prepared from mycelia, primordia and fruit bodies was determined as described (Kim *et al*., 1996). The enzyme activities were assayed at 290 nm using a molar absorption coefficient of 2,800 $M^{-1}cm^{-1}$ by means of a Shimadzu model UV-265 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 μmole of L-ascorbic acid/min.

Examination of the effects of L-ascorbic acid on fruit body formation

Cultures were grown in a Petri dish (10×10 cm) containing 25 ml of the SA medium. Each dish was inoculated with an agar plug removed from stock cultures using a sterile cork borer. The inoculum plug was inverted and placed in the center of the culture dish. The mycelia were grown in the dark at 25±2℃ for 10 days (primary incubation) and then further incubated under the following conditions (secondary incubation): 1) in the dark at 8±2℃; 2) in alternate light and dark periods (8 light h and 16 dark h per day) at 8±2℃; 3) in the dark at 8±2℃, following treatment with L-ascorbic acid, dehydroascorbic acid, reduced glutathione, and dithiothreitol. Cultures growing in the dark were supplied with L-ascorbic acid and other chemicals sterilized by filtration through a nitrocellulose membrane (0.45 μm). Addition of the chemicals was done under red safe light. The formation of primordia and fruit bodies was observed for each of the culture conditions mentioned above. The primordium is a button-shaped structure produced in the fruiting stage, and the fruit body is the mature basidiocarp.

Results

Effect of nutrition, temperature, and light on fruit body formation in different strains of *P. ostreatus*

In order to study the molecular mechanism involved in photomorphogenesis, it was necessary to establish reproducible culture conditions for inducing fruit body formation from vegetative mycelia. The effects of nutrition, temperature and light on fruit body formation were investigated in various strains of *P. ostreatus*. As shown in Table 1, NFFA 2, NFFA 4001, NFFA 4501, and KFCC 11635 strains developed fruit bodies on the defined synthetic sucrose-asparagine (SA) medium when they were incubated in the dark at 25±2℃ and then transferred to the light at 8±2℃ for 17 days (Fig. 1). However, these strains rarely produced fruit bodies when incubated either under continuous darkness or at the constant

Table 1. Effect of nutrition, temperature and light on fruit body formation in various strains of *P. ostreatus*

Strain	2nd culture condition[c]											
	Synthetic medium						Complete medium					
	10±2°C			25±2°C			10±2°C			25±2°C		
	CL[b]	L/D[b]	CD[b]	CL	L/D	CD	CL	L/D	CD	CL	L/D	CD
NFFA 2	+[a]	+	–[a]	–	–	–	–	–	–	–	–	–
NFFA 4501	+	+	–	–	–	–	–	–*[a]	–	–	–	–
NFFA 4001	+	+	–	–	–	–	–	–*	–	–	–	–
NFFA 2M1	–	–		–	–	–	–	–	–	–	–	–
NFFA 2M2	–	–	–	–	–	–	–	–	–	–	–	–
KFCC 11635	+	+	–	–	–	–	–	–*	–	–	–	–

[a] + indicates fruiting; – indicates no response; –* indicates rare fruiting, if any, later than 70 days.
[b] CL, continuous illumination; D/L, alternate 8 h illumination and 16 h dark; CD, continuous dark.
[c] Mycelia were cultivated at 25°C in the dark for 10 days (1st culture) and further incubated under new conditions described above for 20 days (2nd culture).

temperature of 25±2°C. These strains rarely produced fruit bodies on the complex yeast malt extract (YM) medium regardless of the light and temperature. In addition, these strains did not produce fruit bodies on the diluted YM media, where the vegetative mycelia had a reduced growth rate. However, the NFFA 2M1 and 2M2 strains did not fruit either on the YM or SA medium regardless of the temperature and light (Table 1). These results show that *P. ostreatus* needed to be cultivated on a defined synthetic medium, using low temperature treatment and light to have fruit body formation from the vegetative mycelia.

Effect of light on the production of reductones in the development process

The amount of each reductone found in the various light-induced developmental stages was estimated with HPLC coupled with an electrochemical detector. The concenration of L-ascorbic acid in the illuminated mycelia immediately increased, reaching a maximum level of 36.3 μM at 8 h after light exposure, and afterwards it rapidly decreased within 4 h to 2 μM. Beyond this measurement, L-ascorbic acid was not detected throughout the test period. However, L-ascorbic acid was detected in negligible quantities (less than 0.2 μM) in the unilluminated mycelia (Fig. 2). The production of other reductones in the illuminated mycelia fluctuated. These reductones were detected in a small quantity (less than 0.7 μM) during the 9 days from the beginning of the illumination. A burst in the production of EASC, XyloEASC, GlcEASC, MXyloEASC, and MGlcEASC occurred in the illuminated mycelia exposed to light for 11 days, reaching maximum levels of 4.5, 29.6, 41.2, 0.3, and 2.1 μM respectively. Afterwards, the level of these compounds decreased to an undetectable quantity (less than 0.2 μM) within 4 days. After 24 days from the beginning of the illumination, primordia were released from the mycelia and then fruit bodies were formed from the primordia. Another burst in the production of EASC, XyloEASC, GlcEASC, MXyloEASC, and MGlcEASC occurred in the primordia, reaching concentrations of 9.1, 33.6, 54.8, 23.0, and 22.4 μM, respectively. Such bursts in the production of EASC, XyloEASC, GlcEASC, MXyloEASC, and MGlcEASC also occurred in the fruit body, with concentrations at 8.5, 31.6, 42.5, 16.2, and 26.2 μM, respectively. EASC, XyloEASC, GlcEASC, MXyloEASC, and MGlcEASC were detected in a negligible quantity (less than 0.2 μM) in the unilluminated mycelia (Fig. 2). The staining result for reductones present in the mycelia, primordia, and fruit bodies grown under controlled conditions showed that the primordia and fruit bodies were deeply stained and the mycelia around the primordia were faintly stained (Fig. 3). This staining result corresponded quite well with the level of reductones measured by HPLC.

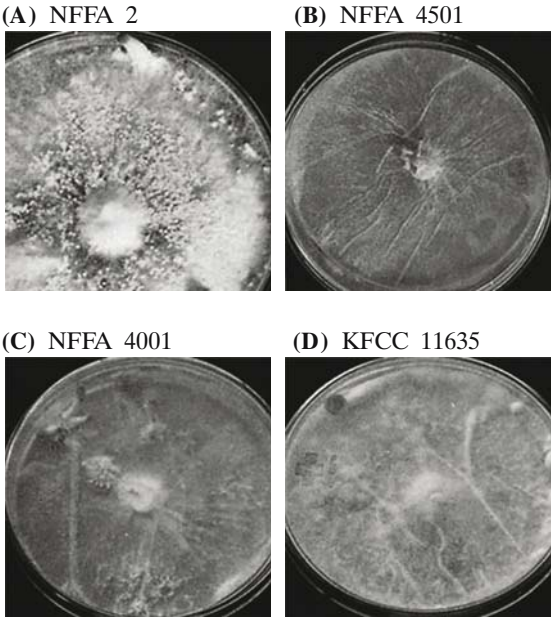

Fig. 1. Fruit body formation in various strains of *P. ostreatus*.

The levels of ascorbate oxidase in the development process

The reductones present in *P. ostreatus* are oxidized by heme-containing ascorbate oxidase with production of hydrogen peroxide. The concentration of hydrogen peroxide produced was quite similar to that of the reductones oxidized (Lee *et al*., 2009). It has been reported that ascorbate oxidase was detected only in the mycelial stage of development in *P. ostreatus* (Kim *et al.*, 1996). The activity of ascorbate oxidase was measured during photomorphogenesis. Ascorbate oxidase was slightly increased in the illuminated mycelia immedi-

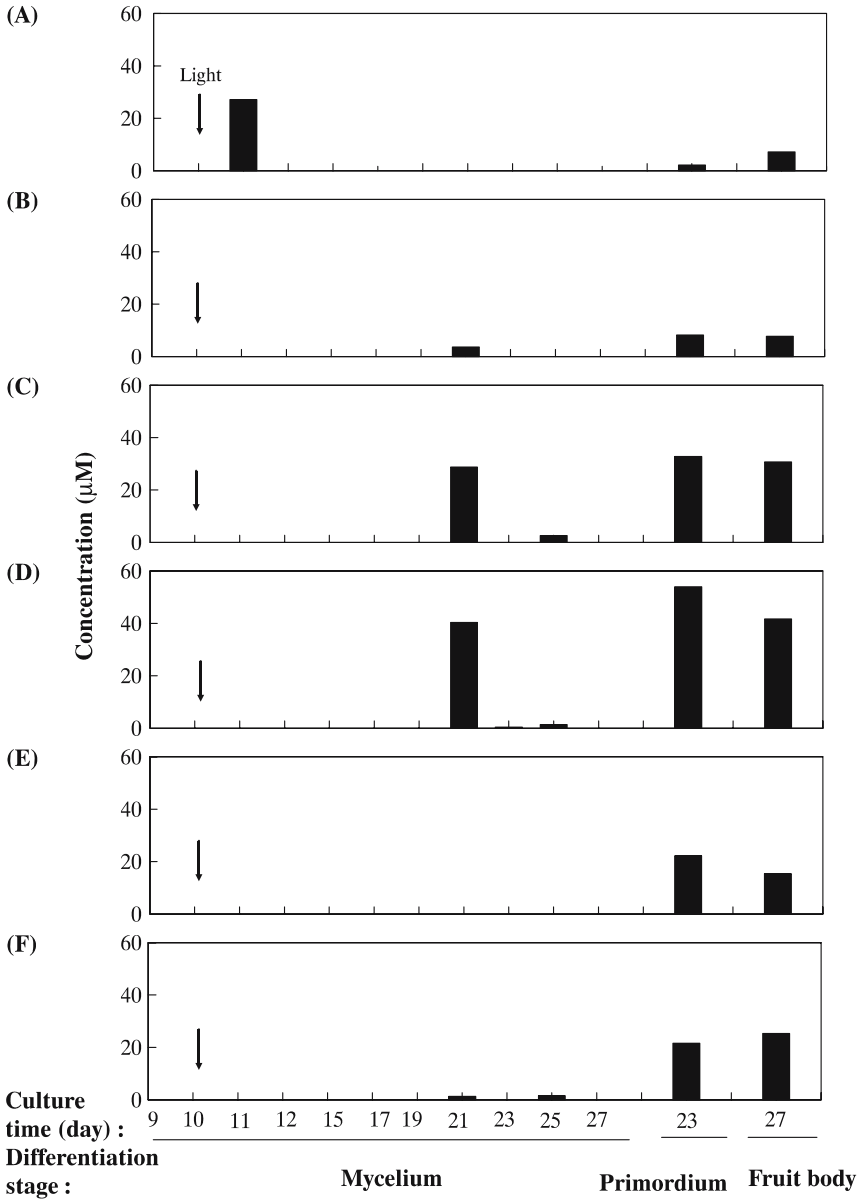

Fig. 2. The content of reductones during differentiation in *P. ostreatus*. Arrow indicates the day when culture plates kept in the dark at 25°C were transferred for secondary incubation at 8±2°C under alternate light and dark periods. (A) L-ascorbic acid, (B) D-erythroascorbic acid, (C) 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, (D) 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, (E) 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, (F) 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid.

ately after the being transferred to the alternating light and dark periods and thereafter it gradually decreased as the cultivation progressed. It was not detected in the primordia and fruit bodies (Fig. 4). This result was confirmed by activity staining on the gel using 2,6-dichlorophenol indophenol (Kim, 1994).

Effect of L-ascorbic acid on differentiation

To demonstrate the implications of production of L-ascorbic acid, glycosides of erythroascorbic acid and their methylated compounds in the differentiation of *P. ostreatus*, the effect of exogenous L-ascorbic acid on the differentiation of *P. ostreatus* was examined. L-Ascorbic acid was applied under red light to the young marginal mycelia grown in the dark for 10 days at 25°C, to final concentrations of 0, 1, 5, and 10 mM. These L-ascorbic acid-treated mycelia were incubated in the dark at 8±2°C for an additional 24 days. The results clearly demonstrated that L-ascorbic acid induced formation of primordia from the mycelia in the dark, in a dose-dependent manner (Figs. 5A and B). The effect of L-ascorbic acid on the formation of primordia was observed at 1 mM and above, and was maximum at 5 mM. Exogenous L-ascorbic acid was oxidized by intracellular heme-containing ascorbate oxidase or metal ions such as Fe^{3+} in the culture medium to produce

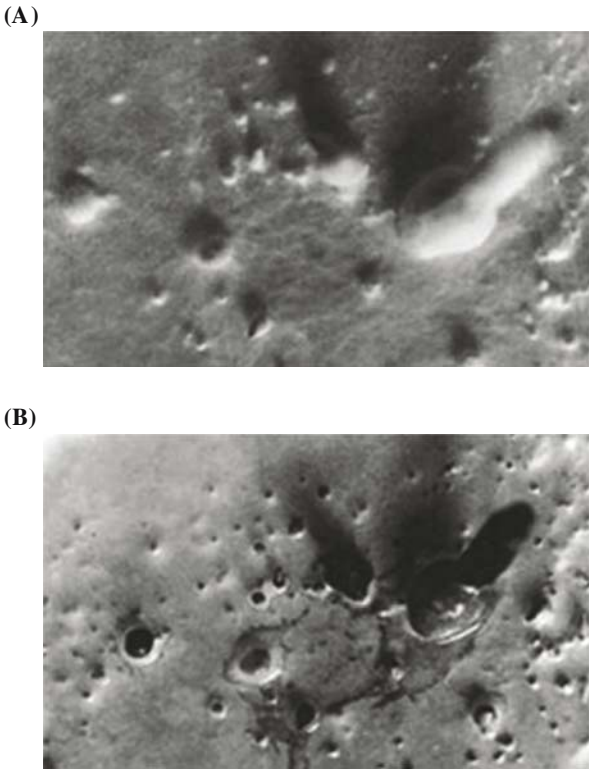

Fig. 3. Detection of reductones in different developmental phases. Before (A) and after (B) staining the cultures with 2,6-dichlorophenol indophenol.

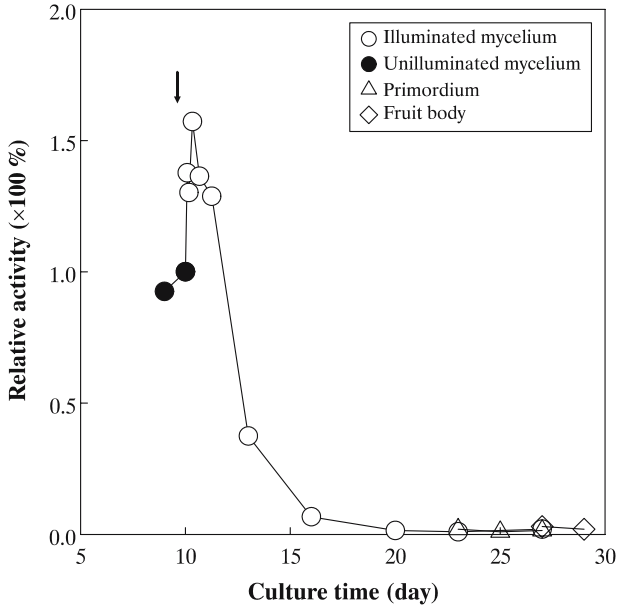

Fig. 4. The activities of ascorbate oxidase during differentiation in *P. ostreatus*. Activity of ascorbate oxidase in the extracts prepared from the mycelia of various ages, primordia and fruit bodies was measured as described in 'Materials and Methods'. Arrow indicates when culture plates kept in darkness at 25°C were transferred to the alternate light and dark conditions at 8±2°C. The data are representative of three separate experiments.

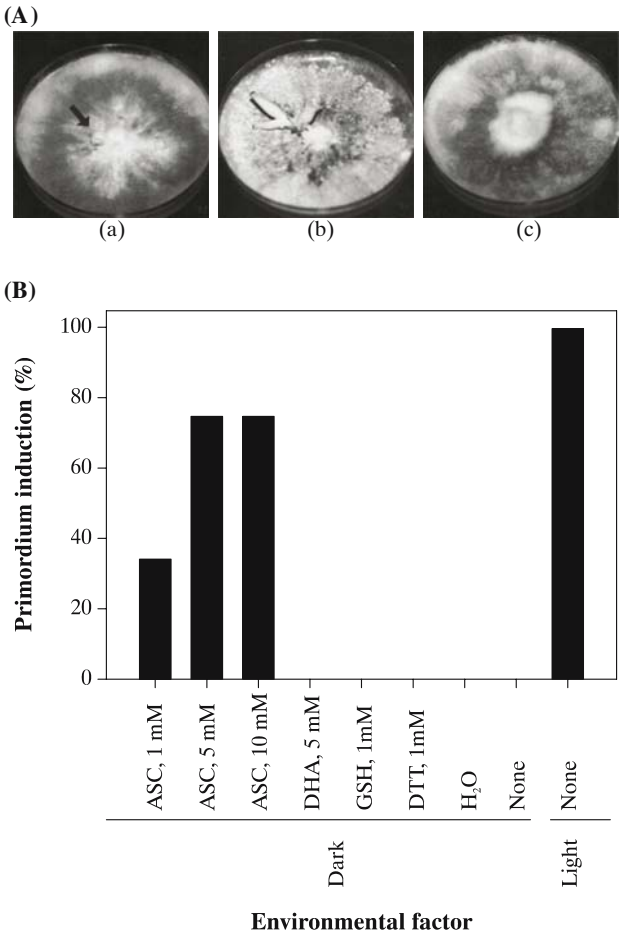

Fig. 5. Effect of L-ascorbic acid on the differentiation of *P. ostreatus*. (A) Primordium and fruit body formation induced by light and L-ascorbic acid. (a) incubated in the dark following treatment with L-ascorbic acid. Arrow indicates the primordium, (b) incubated in alternating light and dark periods, (c) incubated in the dark (control). (B) Effect of L-ascorbic acid on the formation of primordia from vegetative mycelia in the dark. ASC, L-ascorbic acid; DHA, dehydroascorbic acid; GSH, reduced glutathione; DTT, dithiothreitol. The data represent the average of three separate experiments using five Petri dishes for each compound.

dehydroascorbic acid and hydrogen peroxide. The effect of exogenous dehydroascorbic acid, reduced glutathione and dithiothreitol on the development of the fruit body was also examined. These chemicals were applied under red light to the young marginal mycelia grown in the dark for 8 days at 25°C. The chemically treated mycelia were incubated in the dark at 8±2°C for an additional 24 days. Dehydroascorbic acid, reduced glutathione and dithiothreitol were not effective in inducing fruit body formation.

In summary, light stimulated the development of primordia and subsequent fruit bodies from dikaryotic mycelia in *P. ostreatus*. As an immediate response to light, a burst in the production of L-ascorbic acid, glycosides of erythroascorbic acid, and their methylated compounds occurred in the vegetative mycelia. Exogenous L-ascorbic acid could stimulate primordial development in the dark in a dose-dependent manner.

Discussion

In *P. ostreatus*, fruit bodies developed from the vegetative mycelia in the defined synthetic sucrose-asparagine medium when incubated under light. It has been previously shown that L-ascorbic acid, D-erythroascorbic acid and an additional four novel reductones such as 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid are present in the fruit body of the oyster mushroom, *P. ostreatus* (Lee *et al.*, 2009). In this study, we estimated the levels of reductones present during stages in the process of photomorphogenesis and examined their plausible biochemical function in the differentiation of *P. ostreatus.*

The production of the reductones was affected by light and correlated with morphogenesis in the oyster mushroom. After a defined culture period, reductones accumulated in the illuminated mycelium before the initiation of fruit body development. One of the reductones, L-ascorbic acid, when added to the mycelia, induced the initiation of fruiting, though the time required for this initiation was longer than that required by using light. However, dehydroascorbic acid, the oxidized form of L-ascorbic acid did not induce fruit body development. From these results, we can speculate that the reductones produced during the developmental stages may play an important regulatory role in the cellular response of *P. ostreatus* to light. It can be suggested that the reductones produced in the vegetative mycelia prior to the initiation of fruiting play a role in mediating the effect of light on the development of the fruit body in *P. ostreatus* (Fig. 6).

It is known in the other species of fungi that reactive oxygen species participate in the reactions leading to differentiation (Allen *et al*., 1988; Hansberg *et al*., 1993). In *Pyronema domesticum*, aerobic conditions were required for the initial redox reactions in the photoresponse (Moore-Landecker and Shropshire, 1982). Hydrogen peroxide and the other oxidizing agents could replace light in stimulating sporulation in *Alternaria solani* (Carlile, 1974). These findings imply that light may exert its effect by promoting oxidation-reduction reactions which increase the production of reactive oxygen species in the vegetative mycelia of *P. ostreatus* and the reactive oxygen species may trigger the onset of fruit body formation. The reductones may serve as electron sources in the redox reactions. Each reductone was oxidized by the heme-containing ascorbate oxidase to produce equivalent amounts of oxidized compounds and hydrogen peroxide as reaction products (Kim, 1996; Lee *et al*., 2009). Hydrogen peroxide was generated in the culture media supplied with L-ascorbic acid. Exogenous hydrogen peroxide added to the vegetative mycelia also induced initiation of fruiting in the dark (Lee and Kang, unpublished data). Therefore, the reductones may play a role in mediating the light response through hydrogen peroxide which acts as a biological messenger in the initiation process for fruit body formation in *P. ostreatus* (Fig. 6).

The content of glycosides of erythroascorbic acid and their methylated compounds increased again in the primordia and fruit bodies. The results indicate that these compounds, as the storage forms of both sugar and D-erythroascorbic acid, may be utilized for the synthesis of macromolecules needed for cell division, which occurs rapidly during the differentiation of vegetative mycelia into the primordia and subsequent fruit bodies. The sugar moiety could play a role in transporting D-erythroascorbic acid derivatives through the sugar transporters in the cell membrane (Vera *et al*., 1993). The D-erythroascorbic acid moiety of derivatives could act as a carrier of xylose or glucose, which are constituents of cell wall compounds during morphogenesis. They could also act as free radicals (Gonzalez-Reyes *et al*., 1994). These free radicals could activate a membrane redox system that increases the uptake of nutrients required for the development of primordia and fruit bodies.

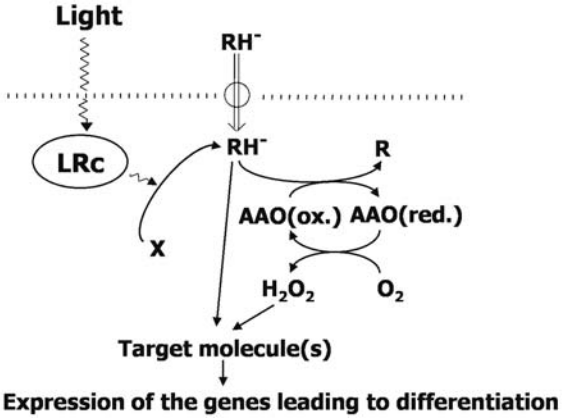

Fig. 6. A proposed scheme showing the effects of light and reductones on the development of *P. ostreatus*. RH^-, reductone; R, oxidized product; AAO, ascorbate oxidase; LRc, light receptor; X, unknown substrate.

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