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**Original Research Article**

# Vitamin C modulates metabolic responses in hemiascomycete riboflavinogenic fungus *Ashbya gossypii*

**S.Kavitha and T.S.Chandra***

Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600 036, India
**Corresponding author*

**A B S T R A C T**

*Ashbya gossypii* is a natural overproducer of riboflavin and a known plant pathogen. Literature reports show a close link between stress and riboflavin overproduction in this organism. However the effect of reductive conditions on the metabolic responses has not been studied in this organism. In the present study different concentrations of vitamin C (2-50 µM) were used to study the effect on growth, total riboflavin levels and extracellular riboflavin secretion. There was a significant increase in biomass on day 3 compared to controls with all concentrations of vitamin C tested, indicating the protective effect of vitamin C. Vitamin C at 5, 20 and 50 µM increased the day 3 secretion of riboflavin to 47.2%, 42% and 44% respectively which was higher than 27.9% in controls. An analysis of the oxidative stress parameters on supplementation of 20 µM Vitamin C showed a decrease in intracellular reactive oxygen species (IC ROS), lipid peroxides (LPX) and membrane lipid peroxides (MLPX) in comparison with controls, indicating its anti-oxidant property. Vitamin C increased superoxide dismutase (SOD), GSH/GSSG ratio, glutathione reductase (GR) and glutathione S transferase (GST) compared to controls. Catalase (CAT) and glutathione peroxidase (GPX) levels remained unaffected by vitamin C. From the above it appeared that vitamin C through its anti-oxidant property protected *A.gossypii* cells, and thus increased biomass. The study is first of its kind to explore riboflavinogenesis and oxidative metabolism under reductive conditions in *A.gossypii*.

**Keywords**

*A. gossypii*; Vitamin C; riboflavin; oxidative stress; GSH metabolism

## Introduction

*Ashbya gossypii* is a plant pathogen and is known to infect cotton plants causing stigmatomycosis. Plants on exposure to a microbial assault could mount an effective response known as oxidative burst. Thus the fungus should host a potential defense mechanism to combat the molecules of oxidative burst particularly, $H_2O_2$. It is to be noted that *A.gossypii* is a natural overproducer of riboflavin and a few literature reports depict a close link between riboflavin production and stress in this organism. Riboflavin production is increased by nutrient limitation (nutrient

stress) and decreased by cAMP supplementation (a negative stress signal) (Schlosser et al., 2007, Stahmann et al., 2001). In an earlier study it was found that low levels of vitamin E and menadione increased riboflavin production and secretion (Kavitha and Chandra, 2009). A recent study showed that *Agyap1* mutants were sensitive to oxidative stress induced by $H_2O_2$ and menadione and exhibited decreased riboflavin production (Walther and Wendland, 2012). In view of the above it appears that riboflavin overproduction in *A.gossypii* could be a part of the stress defense mechanism. However, the metabolic response including riboflavinogenesis has not been explored under reductive conditions. Vitamin C provides the reductive conditions in cells as it is a strong natural anti-oxidant. Vitamin C can scavenge peroxyl radicals, superoxide radicals and singlet oxygen (Jia-Quan, 2008).

Vitamin C plays many important biochemical roles in most organisms including very important cell functions like growth and differentiation (Georgiou, 2001). Apart from the anti-oxidant property of vitamin C, it is also found to promote sclerotial differentiation in *Sclerotina minor* (Georgiou, 2001).

Vitamin C as an anti-oxidant promotes the growth of higher fungi through its effect on` oxidation-reduction potential of cells (Cochrane 1958, Thompson et al., 2001). Vitamin C supplementation inhibited the secondary metabolite aflatoxin B biosynthesis in *Aspergillus flavus*. However in *A.gossypii* the effect of vitamin C on growth, production and secretion of secondary metabolite riboflavin is not explored. Hence the present study explored the effect of vitamin C on growth, riboflavinogenesis and oxidative stress parameters in *A.gossypii*.

GSH could conserve vitamin C *in vivo*, and vitamin C could conserve GSH (Winkler et al., 1994). Hence glutathione metabolism and vitamin C regeneration are linked. The glutathione metabolism is also linked with riboflavin metabolism. Glutathione reductase requires FAD as a cofactor along with NADPH for the reduction of oxidized glutathione to GSH (Christopher, 1991). Therefore the GSH metabolism was explored in presence of vitamin C in this riboflavin overproducer.

## Materials and Methods

Vitamin C (Ascorbic acid), tetramethoxy propane (TMP), thiobarbituric acid (TBA), reduced and oxidized glutathione, glutathione reductase, dithio-bis-nitrobenzoic acid (DTNB) were procured from Sigma-Aldrich, Illinois, USA. 1-chloro 2, 4-dinitrobenzene (CDNB) and other chemicals used for the study were from Sisco Research Laboratories (SRL), Mumbai, India.

### Organism, growth conditions and exposure to stressors

*A.gossypii* culture, NRRL Y-1056 was obtained from NCAUR, Illinois, USA. It was maintained on Yeast-Malt extract agar (YMA) slants of the following composition (g/l); 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g glucose, 20 g agar. Mycelium were grown in Ashbya full medium (AFM) containing (g/l) 10 g casein, 10 g yeast extract, 20 g glucose, 1 g myo-inositol (Altmann-Johl and Philippsen, 1996) at 180 rpm at 30 ºC for all experiments. A preinoculum of 0.5 % grown for 48 h in the above medium was used to inoculate 50 ml of AFM in a 250 ml brown Erlenmeyer flask for all experiments. A stock solution of 5 mg/ml of Vitamin C (MW 176.12) was made in sterile water. From the stock 36 µl

and 90 µl were added to 50 ml of AFM, which corresponds to 20 µM and 50 µM respectively. A 1 in 10 dilution of the stock was made and from the second stock 36 µl and 96 µl were added to obtain a final concentration of 2 µM and 5 µM respectively.

The cells were harvested by centrifuging at 15,000 rpm for 20 mins. Biomass was determined after drying the cells at 98°C to a constant weight as reported earlier (Kavitha and Chandra, 2009). Total and extracellular riboflavin was estimated spectro-fluorimetrically using the ISI standard procedure as described elsewhere (Kavitha and Chandra, 2009). A constant amount of frozen cells were used for cell-free extract preparation using a mortar and pestle as mentioned previously (Kavitha and Chandra, 2009).

**Analysis of non-enzymatic stress parameters**

Reduced glutathione (GSH) was measured by the formation of 5-thio-2-nitrobenzoic acid with Dithiobisnitrobenzoic acid (DTNB), spectrophotometrically, at 412 nm in the protein free cell extract (Kavitha and Chandra, 2009). The protein free extract was made by treating 0.5 ml of cell-free extract with 10 % TCA. The reduced glutathione was expressed as nmoles/mg of protein as described earlier (Kavitha and Chandra, 2009).

The toal glutathione (GSH + GSSG) was measured after the GSSG present in the sample was converted to GSH by the highly specific glutathione reductase and NADPH. The total GSH was estimated similar to that of reduced GSH (Kavitha and Chandra, 2009).

**Lipid peroxidation**

The cell free extract (1.0 ml) was used to determine the lipid peroxide levels. Lipid peroxides like malondialdyhyde reacts with thiobarbituric acid (TBA) to form a red colored adduct with a maximum absorbance at 532 nm. The product formed can be detected with sensitivity using HPLC (JASCO with a PDA detector). A RP-C-18 Hibar 15 column was used for the analysis. Tetramethoxy propane (10 mmol/l-1 in 50% methanol) was used as standard after dilution to 0.5, 1.0, 1.5, 2.0 and 2.5 mM. The mobile phase, methanol-potassium phosphate buffer solution (0.025 mmol/l, pH 6.2; 60:40, v/v), was filtered through a 0.45 µm membrane filter and degassed. The red colored adduct had a retention time of 3.57 min at a flow rate of 0.8 ml/min (Kavitha and Chandra, 2009).

**Membrane Lipid Peroxidation**

The *A.gossypii* cells (1.5g/l wet weight) were suspended in 6.0 ml of osmotic stabilizer (0.2M phosphate buffer containing 0.04 M ammonium sulphate) and 2.0 ml of Novozyme (2 mg/ml) and left at 180 rpm for 2 hrs in 29ºC followed by centrifugation at 2500 rpm for 30 minutes at 4ºC. 8.0 ml of Tris-HCl buffer (pH 7.4) was added to the above mixture and incubated for 1 hr at 4ºC followed by centrifugation at 10,500 rpm for 30 minutes. The supernatant was discarded and the pellet consisting of membranes was suspended in methanol-phosphate buffer. The membrane lipid peroxidation was estimated in the suspension by TBA reactivity followed by detection of the product by HPLC as mentioned above (Kavitha and Chandra, 2009).

**Analysis of enzymatic stress parameters**

The activity of superoxide dismutase (SOD) was measured as the inhibition of the rate of reduction of cytochrome c by the superoxide radical, formed by xanthine-xanthine oxidase system. The reduction was observed at 550 nm by continuous spectrophotometric rate determinations. One unit inhibits the rate of reduction of cytochrome c by 50 % at pH 7.8. 0.1 ml of CFE (containing 1.0 -2.0 mg of protein/ml) was used for the assay (McCord and Fridovich, 1969)

The catalase activity was measured by following the decrease in absorbance at 240 nm on decomposition of $H_2O_2$ as described earlier. One unit of catalase is defined as μmoles of $H_2O_2$ consumed/min (Kavitha and Chandra, 2009). The amount of reduced glutathione consumed for decomposition of hydrogen peroxide was measured for assaying the glutathione peroxidase (GPX) activity as described in an earlier work (Kavitha and Chandra, 2009). One unit of GPX is μM of GSH consumed/min.

Glutathione reductase (GR) was assayed by measuring the formation of NADP, which is accompanied by a decrease in absorbance at 340 nm. One unit of the enzyme is oxidation of 1 μmole of NADPH/min at 25 °C at pH 7.0. 0.1 ml of CFE (containing 1.0-2.0 mg of protein/ml) was used for the assay (Pinto et al., 1984).

The Glutathione S Transferase (GST) catalyzed formation of GS-DNB (a dinitrophenyl thioether), was detected by spectrophotometer at 340 nm. One unit of GST activity is defined as the amount of enzyme producing 1 μmole of GS-DNB conjugate/min. 0.3 ml of CFE (containing 1.0-2.5 mg/ml of protein) was used for the assay (Margareta et al., 1987).

The protein content in the CFE was determined by the method of Lowry *et al* using bovine serum albumin as standard (Lowry et al., 1951).

**Intracellular Reactive Oxygen Species (IC ROS) Quantification**

The cells collected on day 2 (100 mg) were incubated for 2 h with 0.4 mg/ml of dichlorofluorescein diacetate (DCF). At the end of 2 h the cells were sonicated for 7 min with a pulse of 2 sec and centrifuged at 10000 rpm for 10 min to remove cell debris. The ROS were quantified in a spectrofluorometer (Jobin Yvon Flourimeter) with an excitation at 488 nm and emission at 530 nm (Emri et al., 1999). The readings were taken after quenching riboflavin with 10.0 mg of sodium dithionite.

## Results and Discussion

**Effect of vitamin C (2-50 μM) on biomass and riboflavin production**

Different concentrations of vitamin C (2-50 μM) were used to study the effect on growth, total riboflavin and extracellular riboflavin secretion (Table 1). Maximum biomass was attained on day 2 in control cells, but on day 3 with vitamin C supplementation. The day 3 biomass was significantly higher than controls with all concentration of vitamin C tested and was highest with 20 and 50 μM (12.6 g/l). The total riboflavin production did not increase with vitamin C. However there was an increase in secretion of extracellular riboflavin levels with 5, 20 and 50 μM of vitamin C on day 3. The percentage secretion was 47.2%, 42% and 44% respectively which was higher than 27.9 in controls. Since cell lysis ensues from day 4 further increase in extracellular riboflavin

could be attributed to lysis rather than an increase in secretion. Most of the other experiments were done only on day 3 since the biomass was significantly high and the cells were intact without lysis.

Vitamin C is an anti-oxidant and is known to promote growth of higher fungi through its effect on oxidation-reduction potential of cells (Cochrane 1958, Thompson et al., 2001). Vitamin C reduces oxidative stress during growth (Georgiou and Petropoulou, 2001). In view of the above literature it appears that in the present study vitamin C was protective in *A.gossypii* by increasing its biomass. Vitamin C is a well known anti-oxidant and was expected to decrease riboflavin production due to the following reasons. Riboflavin overproduction in *A.gossypii* is linked to stress as nutrient limitation (Schlosser et al., 2007) and oxidative stress are known to increase riboflavinogenesis (Walther and Wendland, 2012). Though in the present study vitamin C did not increase riboflavin production it has not altered the basal production of riboflavin, indicating that it might not be stressful to the organism. Riboflavin secretion was increased by vitamin C, which was surprising, since vitamin C could inhibit lipid peroxidation and thus protects cell membranes (Georgiou and Petropoulou, 2001). The analysis of oxidative stress parameters and lipid peroxidation may reveal the true status within the cells and explain the above observations. Further experiments were done on supplementation of 20 µM vitamin C owing to a significant increase in biomass compared to controls.

**Effect of vitamin C on IC ROS accumulation, CAT and SOD levels on day 3**

The IC ROS accumulation was lower in vitamin C supplemented cells (Table 2) which can be attributed to the anti-oxidant property of vitamin C. Vitamin C did not affect the CAT and GPX levels, but increased the SOD levels significantly compared to controls.

Vitamin C scavenges IC ROS accumulation (Thompson et al., 2001) and due to this it decreases the expression of stress enzymes (CAT and SOD) and Hsp's (Heat shock proteins) (Thompson et al., 2001). In support of the above literature the CAT levels were not increased by vitamin C, surprisingly SOD levels were increased in the present study (Table 2). A significant increase in SOD with a simultaneous decrease in TBARS was observed in *Candida albicans* on supplementation of 2, 10 and 20 mg of vitamin C, indicating that anti-oxidants can cause an increase in expression of SOD in an effort to bring down the levels of ROS (Ojha et al., 2010).

**Effect of vitamin C on LPX and MLPX**

The LPX and MLPX levels were similar to controls (Table 2). The IC ROS levels were decreased by vitamin C and SOD levels were increased and hence vitamin C did not cause peroxidation. ROS causes lipid peroxidation wherein the lipids in the cell membranes are damaged (Jambunathan, 2010). Lipid peroxidation impairs the structural integrity of the membranes (Moradas-Ferreira, 1996). From the results it appears that the membrane has been protected in presence of vitamin C.

However, vitamin C has increased secretion of riboflavin (Table 1) though there was no increase in membrane lipid peroxidation. Vitamin C is known to inhibit ATPase activity, which is involved in active transport (Wang et al., 2003). The vacuolar accumulation of riboflavin is mediated by an active transport involving AgVMA1. A

knock-out of the vacuolar ATPase subunit AgVMA1 resulted in the complete excretion of riboflavin into the medium, since vacuolar accumulation is an active transport mechanism (Forster et al., 2001). Vitamin C could have inhibited the AgVMA1 and probably resulted in increased secretion of riboflavin. However further studies in this aspect is warranted to confirm the above mentioned.

**Effect of vitamin C on GSH and GSH metabolism**

The GSH and GPX levels were not affected by vitamin C, whereas the GSSG levels were decreased. The decreased GSSG levels in turn caused a significant increase in the GSH/GSSG ratio. The GR and GST activity was increased by vitamin C supplementation (Table 3).

The GPX activity was low similar to CAT activity, since the IC ROS levels were low (Table 2). Exogenous $H_2O_2$ supplementation increased CAT and GPX induction (Lee et al., 1995). The low enzyme activity of CAT and GPX depicts that vitamin C lowered IC ROS due to its scavenging property and hence the demand for CAT and GPX decreased.

The GSH levels were similar to control and the GSSG levels were lower which contributed to increase in GSH/GSSG ratio. GSH/GSSG ratios were higher than controls as expected of an anti-oxidant (Table 3). Under stress conditions GSH is utilized to scavenge the ROS directly (Han and Park, 2009).

In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+$+ $e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG) (Han and Park, 2009). Since the GSH levels were not affected and GSH/GSSG ratio was increased the protective nature of vitamin C becomes apparent in the present study.

The increased GR activity could have contributed to decreased GSSG levels (Table 3). GR utilizes NADPH as a cofactor and is increased as a stress protective mechanism (Herrero et al., 2008).

The increase in GST would have been due to the protective effect of vitamin C (Table 3). There was a decrease in GST by vitamin C in *C.albicans* due to its pro-oxidant effect (Ojha et al., 2010). Since in the present study there was an increase in GST activity it appears that vitamin C had more of anti-oxidant effect at the concentration tested in the present study. GST conjugates GSH, to products of oxidative stress and excretes them as a GSH-conjugated molecule. The GST's play an important role in removing products of oxidative stress, specifically the lipid peroxide end products like endogenous unsaturated aldyhydes, quinones, epoxides and hydroperoxides (Masella et al., 2005). GST's are also known to protect the membrane damage by preventing lipid peroxidation caused by ter-BOOH in rat liver (Aniya and Daido, 1994). The increased GST could have protected the membranes from lipid peroxidation.

Though vitamin C has influenced most of the molecules involved in oxidative stress defense and GSH metabolism, it appears to be protective at the particular concentration tested by influencing these molecules positively.

**Table.1** Screening for effect of different concentration of vitamin C on biomass and riboflavin production

| | Day 1 | | | Day 2 | | | Day 3 | | | Day 4 | | | Day 5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Concentration of vitamin C added to medium** | **Biomass g/l** | **Total $B_2$ mg/l** | **EC $B_2$ mg/l** | **Biomass g/l** | **Total $B_2$ mg/l** | **EC $B_2$ mg/l** | **Biomass g/l** | **Total $B_2$ mg/l** | **EC $B_2$ mg/l** | **Biomass g/l** | **Total $B_2$ mg/l** | **EC $B_2$ mg/l** | **Biomass g/l** | **Total $B_2$ mg/l** | **EC $B_2$ mg/l** |
| **Control** | 3.0 ± 0.1 | 10.0 ± 1.0 | 2.3 ± 0.6 | 10.0 ± 0.6 | 134 ± 12.0 | 21.5 ± 4.5 (16%) | 9.0 ± 0.23 | 184 ± 12.3 | 51.4 ± 5.4 (27.9 %) | 7.0 ± 0.6 | 187 ± 14.0 | 75.6 ± 8.0 (40%) | 6.2 ± 0.6 | 187 ± 13.5 | 72.3 ± 4.0 (38%) |
| **2 µM** | 3.0 ± 0.1 | 12.4 ± 1.1 | 3.6 ± 0.7 | 10.8 ± 0.1 | 154 ± 11.0 | 35.3 ± 7.8 (23%) | 11.4 ± 0.4* | 193 ± 14.0 | 57.8 ± 10.0 (30%) | 6.9 ± 0.6 | 197 ± 13.0 | 105 ± 12.0* (53%) | 5.4 ± 0.3 | 198 ± 12.2 | 111 ± 9.8* (56%) |
| **5 µM** | 3.6 ± 0.1 | 13.0 ± 1.3 | 2.9 ± 0.4 | 10.2 ± 0.3 | 127 ± 11.0 | 34.0 ± 6.4 (27%) | 11.2 ± 0.24* | 190 ± 13.4 | 89.7 ± 8.0* (47%) | 7.5 ± 0.1 | 187 ± 11.0 | 110 ± 6.5* (59%) | 5.6 ± 0.1 | 190 ± 11.9 | 116 ± 8.0* (61%) |
| **20 µM** | 3.4 ± 0.4 | 10.0 ± 1.2 | 2.9 ± 0.5 | 10.6 ± 0.7 | 135 ± 10.0 | 39.5 ± 6.0 (29%) | 12.3 ± 0.23* | 191 ± 13.0 | 80.9 ± 10.5* (42%) | 7.4 ± 0.1 | 188 ± 15.0 | 112 ± 8.0* (60%) | 5.7 ± 0.1 | 195 ± 12.0 | 130 ± 8.0* (67%) |
| **50 µM** | 3.3 ± 0.2 | 12.0 ± 1.2 | 2.7 ± 0.7 | 10.5 ± 0.3 | 140 ± 9.8 | 32.4 ± 4.6 (23%) | 12.6 ± 12* | 196 ± 11.0 | 86.0 ± 4.6* (44%) | 8.1 ± 0.1* | 194 ± 14.0 | 107 ± 7.0* (55%) | 6.5 ± 0.1 | 197 ± 11.8 | 117 ± 5.6* (59%) |

Values represent (n≥3±SD) *p-value < 0.05 is significant (Compared with controls). Values in parenthesis indicate % of secretion. $B_2$: Riboflavin, EC $B_2$: Extracellular riboflavin

**Table.2** Effect of vitamin C (20 µM) on oxidative stress parameters on day 3

| Parameters | Control | VC |
|---|---|---|
| **SOD U/mg** | 1.4±0.11 | 2.5±0.33** (1.78) |
| **CAT U/mg** | 294±13.6 | 320±17.3 (1.08) |
| **IC ROS µg/g** | 231±23.2 | 184±12.8* (0.8) |
| **LPX µmoles/mg** | 0.022±0.001 | 0.021±0.002 (0.95) |
| **MLPX µmoles/g** | 0.07±0.00 | 0.06±0.01 (0.86) |

Values represent (n≥3±SD) *p-value < 0.05 is significant (compared with controls). Values in parenthesis indicate % of controls

**Table.3** Effect of vitamin C (20 µM) on GSH metabolism on day 3

| Parameters | Control | Vitamin C (20 µM) |
|---|---|---|
| **GSH nmoles/ mg** | 107±4.5 | 107±12.8 (1.0) |
| **GSSG nmoles/mg** | 15.2±3.8 | 9.4±0.6* (0.62) |
| **GSH/GSSG** | 7.4±2.2 | 11.3±1.1* |
| **GPX U/mg** | 11.7±1.1 | 13.2±1.6 (1.3) |
| **GST U/mg** | 1.4±0.3 | 4.1±0.3*** (2.9) |
| **GR U/mg** | 1.2±0.2 | 2.1±0.1*** (1.8) |

Values represent (n≥3±SD) *p-value < 0.05 is significant (compared with controls). Values in parenthesis indicate % of controls

*Ashbya gossypii* is a plant pathogen and is a riboflavin overproducer. The riboflavin production in *A.gossypii* is linked to stress protection and is understood as a phenomenon to escape the plant defense mechanism. However, this is one of the first reports on the analysis of growth and riboflavin production under reductive conditions. Surprisingly under reductive conditions provided by vitamin C the biomass and riboflavin secretion has significantly increased. This could be an important industrial strategy in product recovery without affecting the cell mass. The analysis of the stress parameters has shown that at the concentration tested vitamin C has modulated the oxidative stress response in a protective manner and could help *A.gossypii* evade environmental stresses. Hence the study has revealed few important strategies to improve product recovery without compromising growth

which could be exploited industrially. Apart from the above the study has for the first time shown how an antioxidant modulates the metabolic responses of *A.gossypii* and hence is of interest in understanding the concepts of basic science.

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