

The Effect of Some Antioxidants on Protein Banding Patterns and Nucleic Acids Content of Three Genera from Dematiaceous Fungi

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Abstract: SDS-PAGE electrophoresis was used to investigate the effect of four antioxidants on protein banding patterns of mats and metabolic solutions of three genera of Dematiaceous fungi viz. *Alternaria tenuissima*; *Curvularia inaequalis* and *Trichoderma aureoviride*. The antioxidants were vitamin E; β -carotene (fat soluble vitamins); vitamin C and folic acid (water soluble vitamins). Quantitative estimation of RNA and DNA was also recorded for the studied fungi under the effect of these antioxidants. The highest molecular weight bands was recorded in *Alternaria tenuissima* and *Curvularia inaequalis* (mats & metabolic solution) treated with vitamin C, while the lowest molecular weight bands was recorded when treated with vitamin E. The water soluble antioxidants induced the accumulation of proteins but the oil soluble antioxidants suppressed this parameter. *Alternaria tenuissima* (mats & metabolic solution) produced lower number of bands than the control sample when grown under the effect of each antioxidant, while *Curvularia inaequalis* mats produced higher number of bands than the control sample. *Trichoderma aureoviride* produced higher number of bands than the control sample under the effect of vitamin C in mats and under the effect of β -carotene, vitamins C and E in metabolic solution. Folic acid had the ability to increase the concentration of nucleic acids "RNA and DNA". On the other hand, β -carotene had the ability to decrease the concentration of nucleic acids of the studied fungi.

Key words: Antioxidant vitamins - vitamin E - β -carotene - vitamin C - folic acid - Protein patterns - Dematiaceous fungi.

INTRODUCTION

The effect of antioxidants on the growth, activity and protein content of fungi was previously investigated by several authors (Bai *et al.*, 2004; Andrea *et al.*, 2008 and Casanova *et al.*, 2008). It was found that in general, Antioxidants produced profound effects on the aforementioned parameters. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols (Reddy *et al.*, 2002; DellaPenna & Last, 2006 and Casanova *et al.*, 2008). Free radicals are highly reactive that either burn a molecule (causes oxidative damage) or pass from one molecule to another turning the recipient into a free radical and finally neutralize the donor (Wei & Lee, 2002 and Andrea *et al.*, 2008).

Antioxidants are essential for the proper regulation of reproduction; protect normal cellular DNA, tissues and organs from potential damage. Vitamin B¹² is a primary part of DNA and RNA synthesis, folic acid helps in synthesis of all DNA & RNA bases, but only in dosages higher than the 400 mg and essential for the division of new cells, vitamin A is required for protein synthesis, RNA synthesis and cell division, Niacin is also needed for DNA synthesis and to repair DNA, vitamin B6 is necessary for Nucleic acid (DNA and RNA) synthesis, Folic acid is a vital co-enzyme required for protein metabolism and the proper synthesis the nucleic acids that maintain the genetic codes and insure healthy cell division. Since folate is needed for DNA and RNA synthesis and DNA repair, deficiencies lower the rate of cell repair and replacement. Manganese also is necessary for DNA/RNA production and is involved with super-oxide dismutase protection against free radical damage. (Wei & Lee, 2002).

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The major goal of this study is to utilize SDS-PAGE electrophoresis technique to investigate the effect of four antioxidants [vitamin E; β -carotene (fat soluble vitamins); vitamin C and folic acid (water soluble vitamins)] on protein banding patterns of mats and metabolic solutions of three genera of Dematiaceous fungi: *Alternaria tenuissima* (Kunze) Wiltshire; *Curvularia inaequalis* (Shear) Boedijn and *Trichoderma Aureoviride* Rifai. Finally, quantitative estimation of RNA and DNA was recorded for the studied fungal species under the effect of these antioxidants.

MATERIALS AND METHODS

Microorganisms and Culture Medium:

Alternaria tenuissima (Kunze) Wiltshire; *Curvularia inaequalis* (Shear) Boedijn and *Trichoderma Aureoviride* Rifai used throughout this work were obtained from our laboratory collection which were isolated previously by Houseny (2005). The cultures were grown on Czepek's Dox broth (Dox, 1910) at 30 °C for 7 days before using.

Preparation of Antioxidants:

Vitamin E and β -carotene (fat soluble vitamins) with the concentration of 10 IU/100 ml medium; vitamin C and folic acid (water soluble vitamins) with the concentration of 10 mg/100 ml medium were used as follows: each antioxidants was inoculated in triplicate of 250 ml Erylnmeyer flasks containing 100 ml of the broth Czepek's Dox inoculated with one ml of spore suspension of each of the studied taxa under sterilized conditions and autoclaved at 121 °C for 15 minutes under 1.5 atmospheric pressure. The flasks were then incubated at 30 °C for seven days. Two Control media were used for the fat soluble vitamins, the first was Czepek's Dox Broth "Dox, 1910" without any additions of antioxidants. The second was the same as the first one except that Tween 80 was added for facilitating the solubility of both vitamins. While for the water soluble vitamins, first control medium only was used.

Determination of protein patterns:

After incubation, the contents of each set of flasks (fungal mats & metabolic solutions) were separated and each type was gathered up and subjected to SDS-PAGE gel electrophoresis described by Laemmli (1970). Protein extraction was conducted by mixing one gm of mats or one ml of the metabolic solution. The mats were then ground to fine powder using a mortar and homogenized with 1 M Tris-HCl buffer (pH 8.8) in clean Eppendorf tube and left in refrigerator over night. Then centrifuged at 12,000 rpm for 20 min. The supernatant of each sample was kept at 0 °C til using. The banding profile of the examined species was photographed while gels are wet. The number of bands was scored then the recording data was computerized and analyzed by Gel proanalyzer version 2.0 soft ware in The Central Lab., Department of Biological Sciences and Geology, Faculty of Education at Ain Shams University. For determination the molecular weights of bands, a calibration curve was prepared by plotting retention factor (R_f) of the marker ($R_f = \text{polypeptide migration distance} / \text{tracking dye migration distance}$) as abscissa against the known molecular weight as ordinate. The unknown molecular weight of each band was estimated by calculating its R_f and comparing its value with the corresponding molecular weight on the standard curve.

Quantitative estimation of nucleic acids:

Total RNA and DNA of the mats were measured according to the method of Morse and Carter (1949). 0.5 g of each fungal mats were ground with 5% ice cooled trichloroacetic acid (TCA) then centrifuged. The precipitate was washed three times with 2 ml of 5 % TCA. The initial supernatant and the washings were combined to form fraction (A) which contains ice-cooled soluble compound. The residue after fraction (A) was extracted three times with five ml of methanol : chloroform (1:2) mixture for complete dilapidation. The lipid free fraction was solubilized in 2.0 ml of 1N KOH at 37 °C for 16-20 h. and the solubilized material was then re-precipitated with 0.4 ml of 6N HCl and centrifuged. The residual precipitate is the protein and DNA fractions while the supernatant contains RNA.

TCA was added to the supernatant to give final concentration of 5 % TCA. After centrifugation, the residue was washed with two ml of 5 % TCA. The supernatant and the washings were combined to give the RNA fraction. The residue after RNA extraction was hydrolyzed in 5.0 ml of 5 % TCA at 90 °C in water bath for 30 min., cooled and then centrifuged. The residue was washed three times with two ml of 5 % TCA. The supernatant and washing solutions were combined to form the DNA fraction.

Ribonucleic acid (RNA) content was estimated colorimetrically by the orcinol reaction as described by Dische (1953). To 0.5 ml RNA extract, three ml of an acid reagent (0.5 ml of 10 % $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ was mixed with 100 ml of concentrated HCl) were added. This was followed by adding 0.2 ml of a freshly prepared 6 % solution of orcinol in 96 % ethanol (100 ml). The mixture was heated in boiling water bath for 20 minutes and its optical density was measured at 660 nm.

Deoxyribonucleic acid was estimated by DPA (diphenylamine) colour reaction as described by Burton (1956). Samples of 1.0 ml DNA extract were mixed with 2.0 ml of DPA reagent (1.5 g of steam distilled diphenylamine were dissolved in 100 ml of redistilled glacial acetic acid, then 1.5 ml of concentrated H_2SO_4 were added and mixed well) let stand at relation between the concentrations of RNA & DNA and the optical density.

RESULTS AND DISCUSSION

The effect of the antioxidants; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of mats and metabolic solutions of the experimental fungi: *Alternaria tenuissima*; *Curvularia inaequalis* and *Trichoderma aureoviride* was investigated. This investigation was carried out by SDS-PAGE electrophoresis which is a technique for measuring the rate and direction of movement of organic molecules (proteins) in response to an electric field (Laemmli, 1970).

The effect of the four antioxidants on the protein banding patterns of *Alternaria tenuissima* mats showed the presence of 17 different bands that ranged between 112.50 and 20.67 KDa. (Fig. 1) and 21 different bands ranging between 48.90 and 1.00 KDa. for metabolic solution of the same fungus (Fig.2). The highest molecular weight bands were recorded in *Alternaria tenuissima* (mats & metabolic solution) treated with vitamin C, while the lowest molecular weight bands were recorded when the same fungus was treated with vitamin E. From the previous results, we found that the water soluble antioxidants induced the accumulation of proteins but the oil soluble antioxidants suppressed this parameter. This may be due to the great ability of fungi for utilizing the water soluble antioxidants and the difficulties when utilizing the oil soluble antioxidants as was previously suggested by several authors (Dalton *et al.*, 1998; Cherubini *et al.*, 2005; Tomader, 2005; Silvera *et al.* 2006; Palumbo *et al.*, 2007; Bjelakovic, 2007. and Kim *et al.*, 2008). *Alternaria tenuissima* (mats & metabolic solution) produced lower number of bands than the control sample when grown under the effect of each antioxidant. Two bands which have molecular weights of (81.00 and 69.67 KDa.) were produced by the control samples (mats) without antioxidants treatments, while three bands having molecular weights of (45.06, 35.82 and 20.30) were produced by the control samples (metabolic solution only) without antioxidants treatments. Kundu, (2003) and Silvera *et al.* (2006) reported that high doses of vitamin E and beta carotene supplements may have pro-oxidant effects. Mascio *et al.* (1991) and Schaffer *et al.* (2005) recorded that in larger doses, vitamin C is an antioxidant, mopping up the oxygen free radicals that destroy cells and cause ageing. It also makes an important contribution to the antioxidant action of other vitamins, particularly vitamin A and vitamin E.

In *Curvularia inaequalis* mats, the electrophoretic protein banding patterns revealed the presence of 37 different bands that ranged between 245.83 and 7.81 Kda. as presented in Fig (3), while in metabolic solution, there were 25 different bands ranging between 98.00 and 3.04 KDa. presented in Fig (4). The highest molecular weight bands was recorded in *Curvularia inaequalis* treated with β -carotene, while the lowest molecular weight band was recorded when treated with vitamin C in both mats & metabolic solution. The results showed that *Curvularia inaequalis* (mats) under the effect of each antioxidant produced high number of bands than the control sample (Fig. 3), while in metabolic solution, lower number of bands were produced than the control sample under the effect of β -carotene and higher number of bands under the effect of folic acid (Fig. 4). The variation in the number of protein bands produced by the tested fungi when exposed to the antioxidants can have some explanation by the fact that the studied fungi experienced a form of stress and so several stress proteins were produced by the fungi as a response to stress. Bai *et al.* (2004) and Eichholzer *et al.*, (2006) clarified that, the stress proteins are formed by microorganisms as a result of a change in the environment such as exposure to heat, radiation or chemicals, so-called stress- susceptible genes are expressed. According to current insights, such proteins can contribute to a protection against detrimental effects resulting from such environmental changes.

In *Trichoderma aurioviride* mats, the electrophoretic protein banding patterns of Tris-HCl buffer (pH 8.8) revealed the presence of 31 different bands that ranged between 138.89 and 30.80 KDa as presented in Fig.(5) and 49 different bands ranging between 287.50 and 2.36 KDa. in the metabolic solution of the same fungus Fig (6). Concerning the influence of β -carotene and folic acid, *Trichoderma aureoviride* (mats) produced lower

number of bands than the control sample, while it produced higher number of bands under the effect of vitamin C but vitamin E has no effect (Fig.5). *Trichoderma aureoviride* (metabolic solution) produced higher number of bands than the control sample under the effect of vitamin β -carotene, C and E, while, under the effect of folic acid there were lower number of bands found than in the control sample Fig (6). The occurrence of additional bands in the PAGE profile may be the result of a synthesis of a new protein controlled by a structural gene, while the variability in band intensity may be associated with an effect of the toxins on the expression of regulatory genes (Mendhulkar, 1993; Georgiou & Petropoulou, 2001; Georgiou *et al.*, 2003). Meanwhile, A protein band observed in the PAGE profile may be composed of more than one polypeptide. This was clearly demonstrated by the detailed investigation by Matta *et al.* (1981).

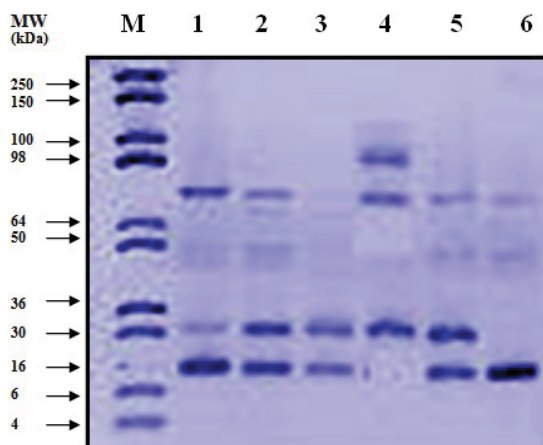


Fig. 1: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Alternaria tenuissima* mats. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

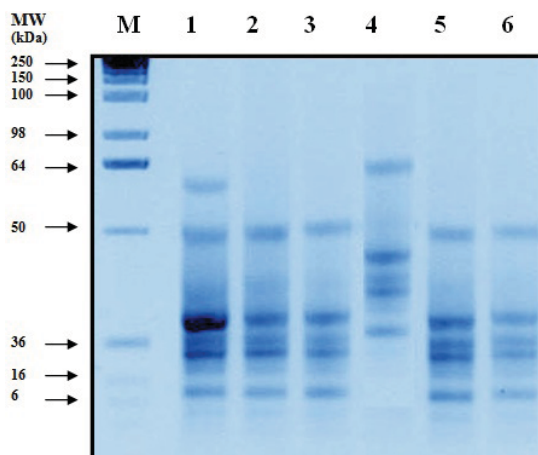


Fig. 2: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Alternaria tenuissima* metabolic solution. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

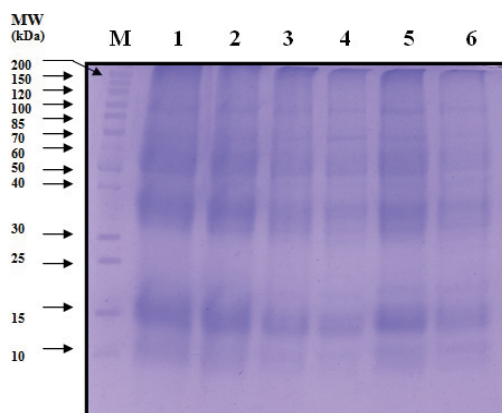


Fig. 3: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Curvularia inaequalis* mats. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

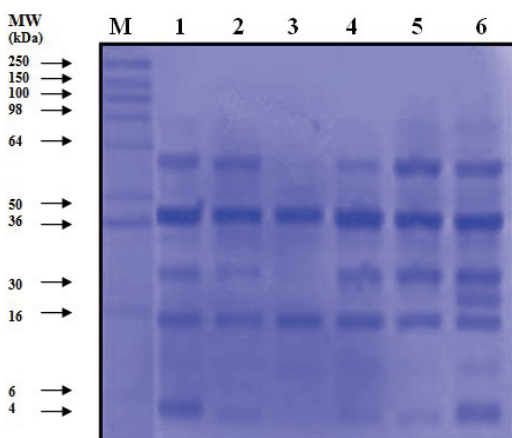


Fig. 4: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Curvularia inaequalis* metabolic solution. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

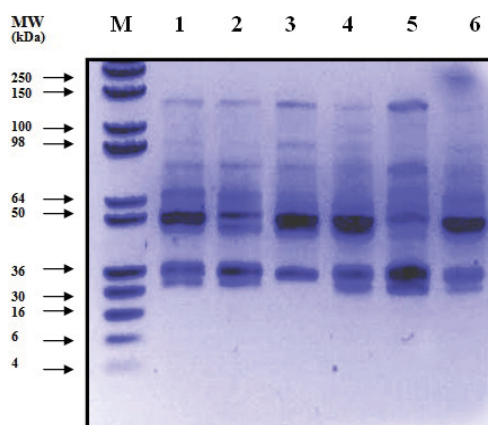


Fig. 5: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Trichoderm aaureaviride* mats. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

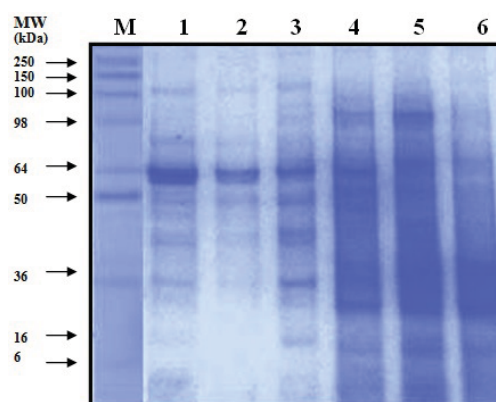


Fig. 6: Effect of antioxidant; β -carotene, vitamin C, vitamin E and folic acid on protein banding patterns of *Trichoderma aureoviride* metabolic solution. M = Marker; 1 = Media without antioxidants; 2 = Media + tween 80; 3 = Media with beta-carotene; 4 =Media with vitamin C; 5 = Media with vitamin E; 6 = Media with folic acid.

The optimum DNA concentrations for *Alternaria tenuissima*; *Curvularia inaequalis* and *Trichoderma aureoviride* were 7.25; 8.22 and 12.80 mg/g mats respectively when adding folic acid to the medium of *Alternaria* and vitamin E to the medium of *Curvularia* and *Trichoderma*. Quantitative estimation of RNA and DNA was recorded for the studied fungal species. The results showed that, the lowest concentration of DNA was recorded in *Alternaria tenuissima* (5.20 mg/gm mats) when grown under the effect of β -carotene (Table 1) and the lowest concentration of RNA was 30.0 mg/gm mats recorded in *Trichoderma aureoviride* when grown under the effect of β -carotene (Table 2). From these results we can conclude that there are some antioxidants such as folic acid "water soluble antioxidant" has the ability to increase the concentration of nucleic acids "RNA and DNA" and consequently the protein banding patterns of fungal mats. On the other hand, there are some antioxidants which have the ability to decrease the concentration of nucleic acids and consequently the protein banding patterns of fungal mats such as β -carotene "oil soluble antioxidant". This may be due to the higher ability of fungi for using water soluble antioxidant than oil soluble antioxidant (Van der Meer *et al.* 2001). Also the results of this work indicate a good correlation between the concentration of nucleic acids, protein accumulation and protein banding patterns.

Table 1: Effect of the experimental antioxidant; beta-carotene, vitamin C, vitamin E and folic acid on DNA concentration of each of the studied fungal mats.

| Fungus | DNA concentration (mg/g mats dry weight of mats) | | | | | | L. S. D. 0.01 = 0.05 = |
|------------------------------------|--|---|--------------------------|----------------------|---------------------|-----------------------|------------------------------|
| | Control 1 (Media without antioxidants) | Control 2 Media) plus Tween 80 without antioxidants) | Media + Beta-carotene | Media + Vitamin C | Media+ Vitamin E | Media + Folic acid | |
| <i>Alternaria tenuissima</i> | 9.54 ± 0.30 | 10.22 ± 0.15 | 5.20 ± 0.13 - HS | 5.60 ± 0.23 - HS | 5.65 ± 0.14 -HS | 7.25 ± 0.14 - HS | 0.35 0.50 |
| <i>Curvularia inaequalis</i> | 7.25±0.24 | 6.45±0.26 -HS | 5.42±0.25 +HS | 7.82±0.15 +HS | 8.22±0.06 -HS | 7.02±0.15 | 0.16 0.23 |
| <i>Trichoderma aureoviride</i> | 11.82±0.16 | 11.88±0.26 | 12.05±0.07 +HS | 12.11±0.14 +HS | 12.80±0.12 +HS | 11.94±0.11 +HS | 0.06 0.08 |

Table 2: Effect of the experimental antioxidant; beta-carotene, vitamin C, vitamin E and folic acid on RNA concentration of each of the studied fungal mats.

| Fungus | RNA concentration (mg/g dry weight of mats) | | | | | | L.S.D. 0.01 = 0.05 = |
|------------------------------------|---|--|-------------------------|---------------------|---------------------|----------------------|----------------------------|
| | Control (Media without antioxidants) | Control 2 Media) +Tween 80 without antioxidants) | Media+ Beta-carotene | Media+ Vitamin C | Media+ Vitamin E | Media+ Folic acid | |
| <i>Alternaria tenuissima</i> | 48.8±0.1 | 50.4±0.1 | 53.6±0.1 +HS | 46.4±0.3 +HS | 39.2±0.4 +HS | 36.0±0.4 -HS | 1.09 1.56 |
| <i>Curvularia inaequalis</i> | 78.4±0.5 | 95.2±0.5 | 128.0±0.5 +HS | 136.0±0.5 +HS | 130.4±0.6 +HS | 133.6±0.3 +HS | 3.85 5.54 |
| <i>Trichoderma aureoviride</i> | 52.8±0.3 | 55.2±0.3 | 30.0±0.5 -S | 48.8±0.3 -HS | 56.8±0.2 +HS | 54.4±0.2 -HS | 1.57 2.26 |

The comparison in case of Beta-carotene and vitamin E was carried out with control 2, but ,in case of vitamin C and Folic acid was carried out with control 1.

HS = Highly Significant.

S = Significant.

NS = Non Significant.

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