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Reactive oxygen participation in gene expression, chlorophyll destruction and lipid peroxidation in oxidative treated leaves of spinach

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Abstract

To find out the role of reactive oxygen species (ROS) on some biochemical traits and gene expression in spinach the experiments were carried out using $AgNO_3$, ascorbate, Dabco and Tiron. For combined treatments, each scavenger was sprayed onto the spinach leaves followed 2h later by the $AgNO_3$ at the fully expand leaf stage. Sampling for chlorophyll and protein measurement, TBARM assay and northern hybridization analysis has been done 48h after the last spray. The results showed the amount of chlorophyll and protein was suppressed by $AgNO_3$ applied in compare with control. The amount of chlorophyll and protein were resumed by using pretreatment of scavenging ROS in combined with $AgNO_3$. The TBRAM results showed $AgNO_3$ treated tissue had the maximum level of TBARM and as it expected the combined treatment As.A+$AgNO_3$ decreased the amount of TBARM significantly. The photosynthetic clones (RBCS and Lhcb) showed significant decreased on RNA levels by treating AgNO3, whereas the transcript was resumed by using pretreatments ROS scavengers specially ascorbate. The rest of the clones used in this study were enhanced-senescence gene expression, for all of them except clones C460 and C30, the expression activity were increased by using $AgNO_3$ treatment and were decreased by pretreatment of ascorbate and tiron in combined with AgNO3. Indicating the role of ROS and practically superoxide ion is important in gene expression.

Keywords: $AgNO_3$, Dabco, Gene expression, Spinach, Tiron

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Introduction

Formation of reactive oxygen species (ROS), can be induced by natural factors such as air pollutant and cosmic radiation (e.g. UV. B) as well as induced factors like, drought salt and chemical stresses. These ROS have dual roles, at high level of concentration they can be quite detrimental and cause some chlorosis and necrosis. Whereas at low level of concentration they act as signaling molecule and could be useful for gene expression and metabolic cell stability (Mackerness, 2000) Silver ion is one of the most toxic metal ions that unfortunately, the amount of this ion is relatively going up in nature (e.g. Water) specially in some countries (Morgan *et al.,* 2005). There are some studies that show that Ag^+ ions inhibit ethylene response pathways. Ethylene is known to regulate many developmental processes in higher plants, including leaf and flower senescence, fruit ripening, stem elongation as well as response to pathogen invasion (Abeles *et al.,* 1992; Morgan and Drew, 1997). Also, there is some information about the role of silver ions in gene expression in plant. Navabpour *et al.,* (2003) tested the effects of different concentration of silver nitrate on the expression a number of senescence-enhanced genes in transgenic *Arabidopsis*. Also, Epple *et al.,* (1995) used silver nitrate on the induction of thionin gene expression in comparison with expression of the *PRla* and *PR5* genes in *Arabidopsis*. Major mechanisms of toxic concentrations actions of heavy metals are covalent ion substitution and interaction with SH groups of proteins, both resulting in the inhibition or inactivation of enzymes (Ciscato *et al.,* 1999; Clijsters *et al*., 1999). The photosynthetic apparatus is affected as a consequence of direct or indirect action of the metal ions. Considerably photosynthesis can be affected by multiple pathways that respond to various autonomous and environmental factors. Metal ions can cause lipid peroxidation in

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photosynthetic membranes and affect both synthesis and degradation of photosynthetic pigments, particularly chlorophyll (Clijsters *et al*., 1999). There is some evidence that Ag^{+} ions could increase the amount of ROS (Navabpour *et al*., 2003). Consequently there is possibility that increased level of ROS by silver nitrate treatment is, at least partly, responsible for the phytotoxic effects of $AgNO_3$ as well as alteration of genes expression. In this study range of $AgNO_3$ concentrations have been used to find out the changes to chlorophyll amount, level of lipid peroxidation and some photosynthetic and defense genes expression.

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Materials and methods

Plant material

Spinach (*spinach oleracea* L. cv. Vienna) was grown in growth room at 20±2 ºC and 14/10 photoperiods, the humidity adjusted at 70±5%.

Chemical treatments

A range of concentrations of each treatment was tested initially to identify the optimum level to use. Final experiments were carried out using 2mM silver nitrate, 20mM sodium ascorbate (general ROS scavenger), 30mM Dabco (singlet oxygen scavenger) and 3mM Tiron (superoxide ion scavenger). All chemicals were obtained from sigma. For the combined treatments, the first treatments (each scavenger separately) was sprayed onto the leaves followed 2h later by the $AgNO_3$ treatment. Sampling for chlorophyll and TBARM assay has been done 48h after the last spray on fully expand leaf stage. For the RNA extraction, similar spray treatments were carried out at the same stage.

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RNA isolation

Plant material (5 g) was ground to a fine powder in liquid N_2 using a prechilled mortar and pestle. The frozen powder was transferred to a 50 ml polypropylene tube containing 10 ml phenol and 15 ml extraction buffer (100 mM Tris-HCl pH 9, 200 mM NaCl, 5 mM dithiothreitol, 1% (w/v) sarcosyl, 20 mM EDTA) and mixed gently. The mixture was centrifuged at 3,000 rpm for 10 min. The upper aqueous layer was extracted two or three times with an equal volume of chloroform, until it became clear. The solution was transferred into a 30 ml Corex tube and adjusted to 2 M LiCl by addition of 1/3 volume of 8 M LiCl and left overnight at 4°C. Precipitated RNA was pelleted by centrifugation at 10,000 rpm, 4°C, for 10 min. The pellet was washed twice with 1.5 ml of 2 M LiCl and recentrifuged each time. The pellet was dissolved in 1.5 ml water. A 10 µl aliquot was diluted with 990 µl water, and used to evaluate RNA concentration and purity by spectrophotometry. The remainder of the solution was mixed with 1/10 volume 3 M sodium acetate pH 5.2 and 2.5 volumes 100% ethanol in a Corex tube, and left at -70°C for 30 min. Precipitated RNA was pelleted by centrifugation and washed in 70% ethanol, and after air drying, dissolved in sterile distilled water at a concentration of 2 µg/µl.

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Northern hybridization analysis

RNA was transferred onto a Hybond N^+ (Amersham) nylon membrane with 0.05 M NaOH as the transfer solution. Transfer occurred overnight and then the filter was removed and washed in 2×SSC. The probe was denatured in a boiling water bath for 4 min, it was then added to hybridization buffer and mixed thoroughly. The filter was replaced in the box and incubated on a shaker at 65ºC overnight. The hybridization

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solution was removed and the filter rinsed in 2× SSC/ 1% SDS, and in some cases in 0.2× SSC/ 1% SDS, and washed twice for 20 min at 65ºC. The filter was dried briefly on filter paper, wrapped in Saran wrap and exposed to X-ray film (Kodak X-OMAT/AR) at -70ºC.

Measurement of thiobarbituric acid reactive material (TBARM)

TBARM (of which malondialdehydes (MDAs) are considered to be a significant component) was measured using an assay modified from Hagege *et al.,* (1990). Plant material (0.5 g) was homogenised with 1 ml trichloroacetic acid (10% w/v). The homogenate was washed with 10 ml acetone, vortexed then centrifuged at 4,750 rpm for 15 min. The pellet was washed in 5 ml acetone, vortexed and then centrifuged at 4,750 rpm for 10 min (4 times). The pellet was dried under nitrogen and incubated at 100°C for 30 min with 3 ml H_3PO_4 (1%) and 1 ml thiobarbituric acid (0.6%). The reaction was terminated by rapidly cooling the tubes on ice. Butan-1-ol (3 ml) was added and the mixture vortexed then centrifuged at 5,500 rpm for 20 min to achieve separation of the phases. Absorbance of the aqueous phase was measured at 532 nm and 590 nm using a Uvikon 930 Spectrophotometer (Kontron Instruments, Watford, UK).

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Chlorophyll Determination

Chlorophyll determinations were made on leaf samples based on the method of Porra *et al*., (1989). Spinach leaves (0.3 g) were extracted in 10 mL of 80% (v/v) acetone. After centrifugation, absorbance (*A*) was read at 646.6, 663.6 and 750 nm on a Uvikon spectrophotometer (Kontron Instruments).

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Protein Determination

Protein was extracted from spinach leaves (0.5 g) in 1.5 mL of extraction buffer (50 mm Tris [tris(hydroxymethyl) aminomethane]-HCL, pH 7.5, 2 mm EDTA, pH 8, 0.04% [v/v] mercaptoethanol). Samples were centrifuged at 12,000*g* for 20 min at 4°C, and proteins determined using a Coomassie protein assay reagent (Pierce Chemical, Rockford, IL) based on a modified Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

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Result and discussion

Changes in chlorophyll and protein

Chlorophyll and protein levels showed a sharp decline in spinach leaves treated by $AgNO_3$ (Fig. 1). Whereas using the combined treatment (As- A+ $AgNO_3$) (Fig.2), that pretreatment of ascorbic acid before $AgNO_3$ was significantly resumed the amount of chlorophyll and protein. It should be mentioned that by spraying ascorbic acid alone on leaves, no changes happened on the amount of chlorophyll and protein levels (Fig.2). Ascorbate is an efficient quencher of number of different ROS (Sturgeon *et al.,* 1998). To determine which particular ROS (singlet oxygen $1/2O_2$ or superoxide ion $O^{.-}{}_2$) was more effective on chlorophyll and protein levels.

Two specific scavengers have been used as a pretreatment 2h before $AgNO_3$ sprayed. The results showed chlorophyll and protein destruction was inhibited by Tiron (superoxide scavenger), whereas pretreatment of Daboco (singlet oxygen scavenger) showed no visible effect. Despite there is some speculation that chlorophyll decline by $AgNO_3$ applied, can be accrued due to ion substitution as case of Zn (Ciscatio *et al.,* 1999), but the above result strongly showed that superoxide produced by $AgNO_3$ is

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quite effective for chlorophyll reduction. Similar result has been reported in this regard (Shimazaki *et al.,* 1980).

TBARM assay result

This assay is one the quick way with good accuracy to find out using $AgNO_3$ on spinach leave resulted to oxidative stress. Oxidative stress results in an increase in lipid peroxidation and the extent of this can be measured by malondialdehydes, (The end and stable product of lipid proxidation). The results showed AgNO3 treatment caused in significant increase in TBARM levels (Fig.2). Indicating there is good possibility for increasing ROS levels by $AgNO_3$ treatment. This is supported by significant decline on TBARM that resulted in combined treatment (As- A+ $AgNO_3$). Interestingly, quite similar to chlorophyll and protein results here the decrease of TBARM for Tiron + $AgNO_3$ was greater than Dabco + AgNO3 which either mean the more activity of superoxide rather than singlet oxygen or more efficiency of Dabco than Tiron to control the effect of singlet oxygen than superoxide respectively. This result has supported by some studies (Navabpour *et al.,* 2003; Mackerness, 2000).

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Gene expression analysis

A number of cDNA clones, have been analyzed using northern blot technique these clones were included photosynthetic genes and some defense and senescence enhanced expression genes. Two photosynthetic genes including RCBS (Ribulose bisphosphate) and Lhcb (light harvesting complex binding proteins) showed significantly decreased levels of expression by $AgNO_3$ treatment applied. There were very similar expression pattern for both genes by applying all treatments correspondingly. As it was expected,

the expression of these genes have resumed fairly by spraying pretreatment of scavengers particularly ascorbic and Tiron. Indicating these genes was generally induced by ROS and specifically superoxide. There have been a numbers of reports to indicate that degradation of stromal proteins such as Rubisco can be initiated non enzymatically by reactive oxygen species (ROS) when plant are involved in stress condition (Ishida *et al*., 1999). Degradation of thylakoid proteins such as Lhcb appears to follow a different route. This protein exists as a pigment – protein complex with chlorophyll and its degradation requires the parallel detoxification of released chlorophyll (Thomas and Donnison, 2000).

Catalases in plants have mainly been associated with the removal of H_2o_2 in peroxisomes. The major source of H_2o_2 in peroxisomes is various oxidases include acyl-COA oxidase, alycolate oxidase and urate oxidase (Willekens *et al.,* 1994). Chloroplasts contain the majority of superoxide dismutase (SOD) activity and about half of the cellular ascorbate peroxidase (APX) activity. SOD enzyme can convert superoxide ion to H_2o_2 that to be scavenged by ascorbate peroxidase (Foyer *et al.*, 1994). Our results showed all three genes (CAT, SOD, APX) were senescence enhanced expression and up- regulated by $AgNO_3$ applied. Interestingly using the combined treatments giving lower levels of transcript, specially for As- A+AgNO3 and Tiron+$AgNO_3$ indicating the up–regulating of these genes were induced by ROS (in particular superoxide). The results of chlorophyll and particularly TBARM strongly supported this. Plant subjected to phytotoxic concentration of heavy metals generally demonstrate symptoms of oxidative stress and develop defense reactions particularly up–regulated genes expression via building up ROS (Ciscato *et al.,* 1999; Jabs *et al.* ,1996).

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Metallothioneins (MTS) are defined as low- MW cys – rich proteins that bind heavy metals. MTS are widely distributed in eukaryotic and prokaryotic organisms (Robinson *et al.,* 1993). In plants, a strong correlation has been observed between MT RNA level and tolerance to heavy metals toxicity, suggesting a role in metal homeostasis in plants (Murphy and Taiz, 1995). Our result showed MT clone has enhanced expression during senescence as well as responded to $AgNO_3$ quite sharply. Using antioxidant treatments (As–A, Dabco and Tiron) in combined with silver nitrate, significantly decreasing the level of MT expression. However, none of the antioxidant was effective as ascorbic and followed by Tiron. These results indicate that $AgNO_3$ treatment of plant tissue causes the generation of a mixture of ROS.

Most of the senescence- enhanced genes tested in this study also showed induced expression. Clone 94 which encodes the pathogenesis related protein (PRla) (Hanfrey *et al.*, 1996), showed very similar pattern of expression to MT. This has been reported in transgenic *Arabidopsis* as well (Navabpour *et al.,* 2003).

However, two clone including C460 and C30 (encode Glutamine synthetase and Ferritin respectively) induced expression during senescence were not expressed in tissue treated with silver nitrate and this indicate that, although the oxidative process has many parallels to senescence it is not necessary to be in same pathways.

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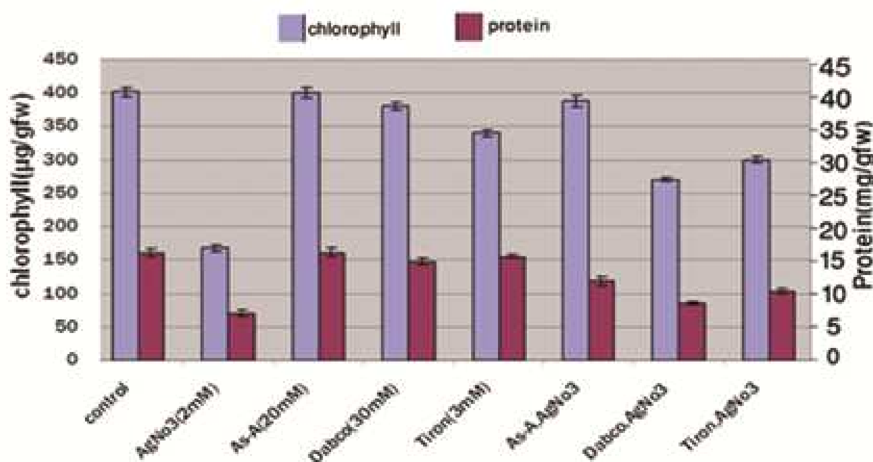

Fig. 1- Chlorophyll and protein changes of spinach treated leaves. All treatments were sprayed on leaves at fully expand leaf stage. For the combined treatments, the first treatments (scavenger) were sprayed followed the second treatment ($AgNO_3$) 2h later samples were taken 48h after the last spray was carried out. Bar shows standard error (n=4).

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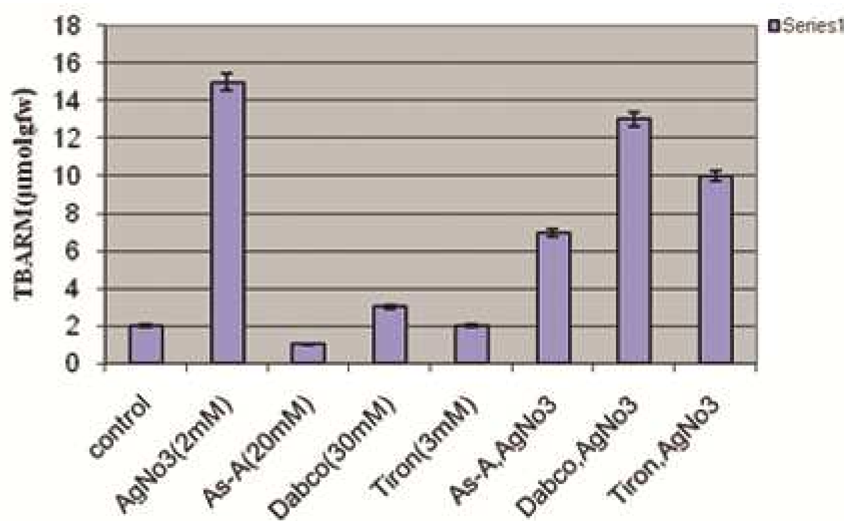

Fig. 2- TBARM changes of spinach treated leaves. All treatments were sprayed on leaves at fully expand leaf stage. For the combined treatments, the first treatments (Scavenger) were prayed followed the second treatment (AgNO3) 2h later. Samples were taken 48h after the last spray was carried out. Bar shows standard error (n=4).

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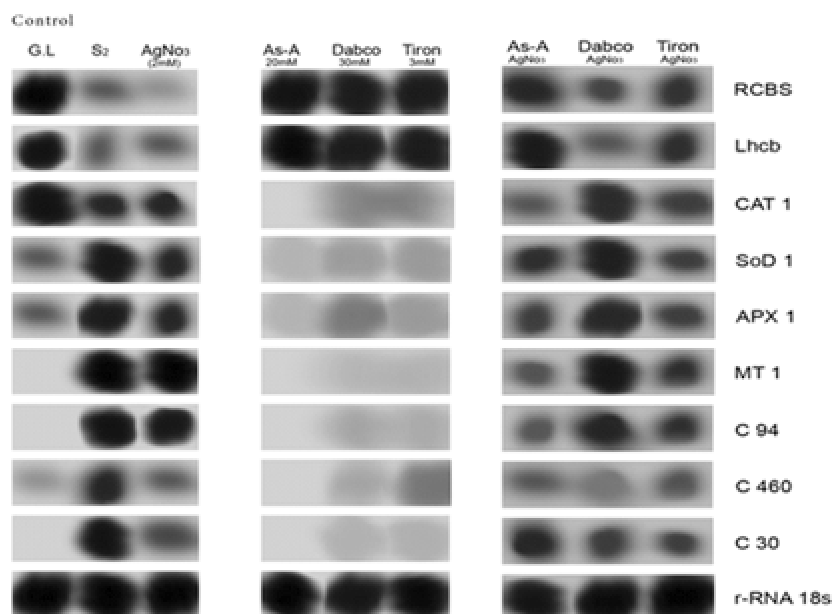

Fig. 3- Northern hybridization analysis. Samples have been taken from spinach treated leaves 48h after the last spray for RNA isolation. Ten micrograms RNA were separated on a denaturing agarose gel, blotted to nylon membrane and probed with ^{32}P labeled gene fragments. Control (Green Leaf-G.L) sample with no spray treatment. Senescent (S2) RNA was isolated from yellowing leaves from mature flowering plants.

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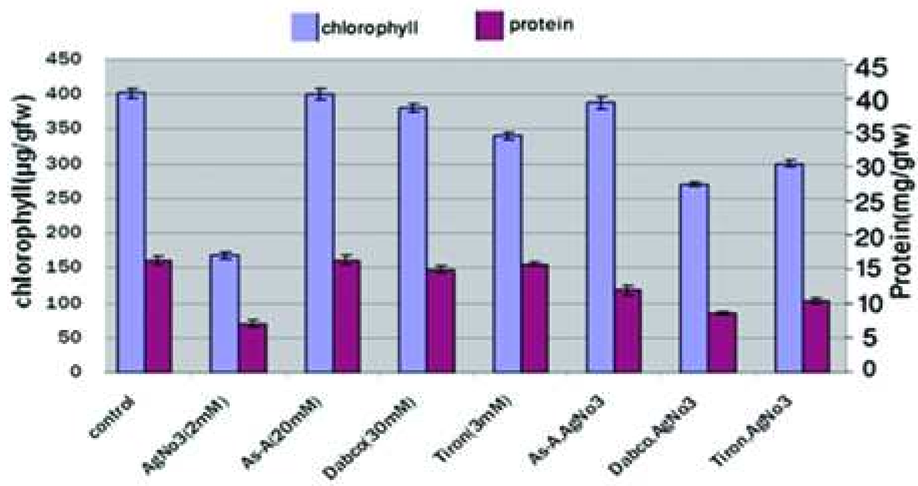

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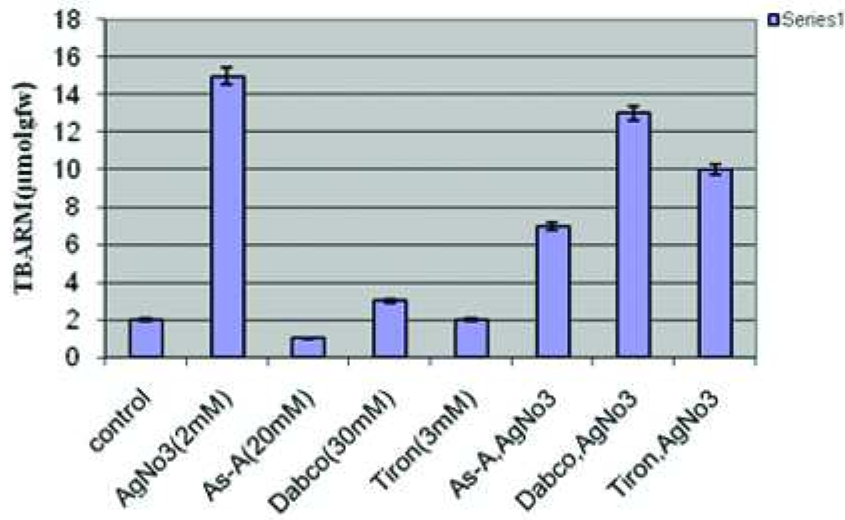
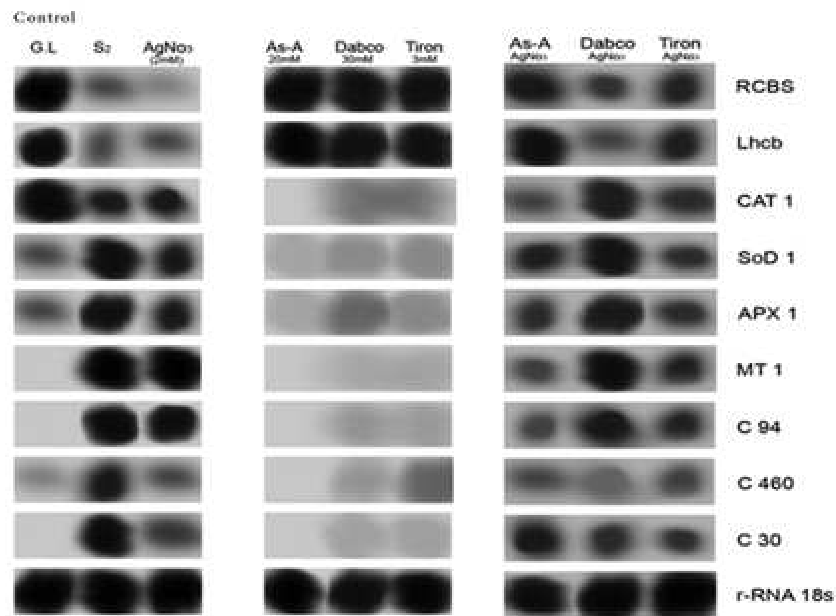


127x73mm (96 x 96 DPI)

For Peer Review



127x87mm (96 x 96 DPI)

Review