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Membrane Degradation, Accumulation of Phosphatidic Acid, Stimulation of Catalase Activity and Nuclear DNA Fragmentation during 2,4-D-Induced Leaf Senescence in Mustard

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We investigated 2,4-D-induced leaf senescence in young mustard seedlings. A set of morphometric, biochemical and molecular parameters were analyzed to characterize senescence markers. In accordance with earlier reports, chloroplast-membrane degradation marked the early phase of leaf senescence based on the analysis of the galactolipid fraction. Degradation of grana occurred earlier to that of the envelope, as revealed by the relative level of their specific galactolipids, namely, monogalactosyl diglyceride and digalactosyl diglyceride. Phospholipids showed extensive degradation resulting in the accumulation of lyso-derivatives of major phospholipids and phosphatidic acid (PA) in senescing leaves. Catalase activity was stimulated by 2,4-D and reflected scavenging of reactive oxygen species. Nuclear DNA degradation, a previously known death signal that represented a point of no return from progression of senescence, occurred late on the 4th day subsequent to 2,4-D supplementation. $AgNO_3$, an inhibitor of ethylene biosynthesis, inhibited leaf senescence by ca. 54% based on PA content. Involvement of 2,4-D, ethylene and abscisic acid in leaf senescence is discussed in relation to hormonal interplay.

Keywords: $AgNO_3$, 2,4-D, Indian mustard, membrane lipids, nuclear DNA, phosphatidic acid

Plant senescence represents a form of programmed cell death (PCD; Greenberg, 1996; Pennel and Lamb, 1997). Phytohormones such as auxins, ethylene and abscisic acid (ABA) and growth factors such as jasmonic acid and several biotic and abiotic factors are known to induce senescence. Senescence involves the differential expression of several metabolic events that are either upregulated or downregulated in a sequential manner (Thomas and Stoddart, 1980; Quirino et al., 2000). These include the degradation of photosynthetic pigments, activation of reactive oxygen species (ROS)-scavenging systems such as catalases, peroxidases and superoxidase dismutases, activation of phospholipases such as phospholipase D (PLD), phospholipase C and phospholipase A_2 and nuclear DNA (nDNA) fragmentation.

Among the factors that induce senescence, auxins are known to have a dual role, both as an elongation factor as well as a senescence factor, in a concentration dependant manner, especially at their site of action (Hansen and Grossmann, 2000). Relatively low concentration of auxin is known to induce cell elongation whereas concentrations beyond a threshold level is senescence inducing. The threshold level for auxin-induced senescence is known to vary even in the same plant system depending on the type of auxin employed, e.g. natural auxins as well as their synthetic analogs such as 2,4-D, NAA, picloram, dicamba, and etc (Hansen and Grossmann, 2000; Quirino et al., 2000). Possible involvement of auxin

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; DGDG, digalactosyl diglyceride; 2,4-D, 2,4-dichlorophenoxyacetic acid; HSR, hypersensitive response; IAA, indole-3-acetic acid; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; MGDG, monogalactosyl diglyceride; nDNA, nuclear DNA; PA, phosphatidic acid; PC, phophatidylcholine; PCD, programmed cell death, PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLD, phospholipase D; PS, phosphatidylserine; ROS, reactive oxygen species; SAG, senescence associated genes; SL, sulpholipid

degrading enzymes and also formation of several conjugated forms of auxin are considered to be involved in maintaining a metabolically active pool of auxin (Grossmann et al., 1996; Hansen and Grossmann, 2000). Conjugated form of auxins is known to be more stable as compared to unconjugated forms against auxin degrading enzymes. This in turn is suggested to be a regulatory mechanism in maintaining auxin homeostasis during different developmental stages. Senescence induction by auxins is due to hormonal interplay involving two other phytohormones, namely, ethylene and ABA (Chae et al., 2000; Hansen and Grossmann, 2000). Auxin induced ethylene synthesis and subsequently synthesis and accumulation of ABA has been reported in a few plants (Eliassion, 1975; Scheltrup and Grossmann, 1995; Grossmann et al., 1996). Especially, ethylene biosynthesis was shown to be stimulated due to over expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, the rate limiting enzymes in ethylene synthesis pathway (Chae et al., 2000; Hansen and Grossmann, 2000). This in turn was shown to regulate ABA synthesis by involving ethylene-mediated signal transduction pathway (Wang et al., 2002). Recently, hormonal interplay involving auxin and cytokinin has also been reported in *Arabidopsis thaliana* (Nordstrom et al., 2004). Thus, hormonal interactions are known to be a common mechanism in the regulation of different developmental responses in plants including leaf senescence (Hansen and Grossmann, 2000).

The present study was undertaken to characterize morphometric, biochemical and molecular events in relation to changes in membrane lipid composition, catalase activity and nDNA profile in agarose gels, that have significant role to play during 2,4-dichlorophenoxyacetic acid (2,4-D)-induced leaf senescence. Supplementation of $AgNO_3$, an inhibitor of ethylene biosynthesis, along with 2,4-D, to the seedlings of mustard was also undertaken to pinpoint the intermediary role of ethylene in 2,4-D-induced leaf senescence. Results of the present study showed that lipolytic products of major phospholipids, namely, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) accumulated in the senescing tissue. In addition to the earlier known membrane degradative process that are associated with senescence, activation of fatty acyl hydrolase(s) that act on the major phospholipids, resulting in the production of lysophosphatidylcholine (LPC) and lyso-phosphatidyletha-

Table 1. Characterization of morphometric parameters and total chlorophyll content due to 2,4-D-induced leaf senescence and senescence arrest by $AgNO_3$ in mustard seedlings.

Treatment	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Specific growth		Shoot weight (mg)	Shoot wt: specific shoot growth ratio	Total leaf chlorophyll (mg g^{-1} fr wt)
				Shoot	Root			
Control-H_2O	3.26 ± 0.08	1.85 ± 0.05	5.11	0.64	0.36	20 ± 0.6	31	0.69 ± 0.06
Control-0.1% acetone	3.22 ± 0.07	1.88 ± 0.04	5.09	0.63	0.37	20 ± 0.5	31	0.65 ± 0.05
300 μM 2,4-D	3.72 ± 0.10	2.09 ± 0.05	5.81	0.64	0.36	21 ± 0.7	33	0.63 ± 0.03
400 μM 2,4-D	2.90 ± 0.08	1.61 ± 0.04	4.51	0.64	0.36	17 ± 0.6	26	0.45 ± 0.02
500 μM 2,4-D	2.37 ± 0.05	1.31 ± 0.06	3.68	0.64	0.36	14 ± 0.6	21	0.28 ± 0.02
600 μM 2,4-D	2.38 ± 0.05	1.32 ± 0.04	3.70	0.64	0.36	14 ± 0.6	22	0.27 ± 0.04
500 μM IAA	2.70 ± 0.08	1.50 ± 0.04	4.20	0.64	0.36	16 ± 0.6	25	0.44 ± 0.04
500 μM 2,4-D + 0.05 mM $AgNO_3$	3.09 ± 0.09	1.63 ± 0.03	4.72	0.65	0.35	20 ± 0.7	30	0.59 ± 0.03
500 μM 2,4-D + 0.10 mM $AgNO_3$	2.62 ± 0.06	1.48 ± 0.04	4.1	0.64	0.36	15 ± 0.6	24	0.42 ± 0.04
500 μM 2,4-D + 0.15 mM $AgNO_3$	2.64 ± 0.06	1.45 ± 0.05	4.09	0.65	0.35	16 ± 0.7	24	0.45 ± 0.05
500 μM 2,4-D + 0.20 mM $AgNO_3$	2.63 ± 0.08	1.47 ± 0.06	4.10	0.64	0.36	16 ± 0.6	24	0.44 ± 0.03
500 μM 2,4-D + 0.25 mM $AgNO_3$	2.57 ± 0.07	1.45 ± 0.03	4.02	0.64	0.36	15 ± 0.7	24	0.41 ± 0.04

2,4-D, IAA, and $AgNO_3$ at indicated concentrations individually and in specified combinations were supplemented to 4 d old seedlings. Determination of various parameters was carried out in seedlings and leaves collected 4 d subsequent to supplementation of experimental solutions.

nolamine (LPE) have been highlighted in the present study.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of Indian mustard (*Brassica juncea* L.) were obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Healthy and uniform size seeds were germinated on a thin pad of absorbent-cotton in 130 mm diameter petri dishes. Seedlings were grown in a growth chamber under white light from fluorescent tubes at 1200 μW cm^{-2} at plant level, with a photoperiod of 16L/8D, 80% humidity and at 25±1°C. Seedlings were irrigated with double distilled water up to 4 d and subsequently treated with experimental solutions. Subsequent to 4 d, irrigation of the seedlings was done either with water (water control), 0.1% aqueous acetone (acetone control) or experimental solutions, as mentioned in the results. Twenty ml of experimental solutions of 2,4-D, indole-3-acetic acid (IAA), and $AgNO_3$ were supplemented only once to 4 d old seedlings at indicated concentrations individually and in specified combinations (Table 1). Subsequent to the commencement of experimental period, irrigation volume was kept at 40 mL per day and the seedlings were irrigated twice daily, 20 mL each time at evenly timed intervals. Acetone control was included in all the experiments wherever IAA or 2,4-D was supplemented to the seedlings, since these auxins were prepared in 0.1% aqueous acetone (Hansen and Grossmann, 2000). Age of plants was indicated as 0 to 4 d corresponding to initial period of germination and seedling growth in distilled water. Subsequent to commencement of experimental treatments, plant age was designated as 0 to 4 d corresponding to the growth of seedlings in experimental solutions.

Determination of Morphometric Parameters

Seedling length represents the additive value of shoot length and root length. Specific growth of shoot and root were derived from the length of shoot and root respectively, in relation to seedling length.

Lipid Analysis

Lipid extraction was carried out according to the procedure of Bligh and Dyer (1959). Lipid analysis was carried out using a two-step chromatographic procedure involving silica gel column chromatography and TLC as reported elsewhere (Sathishkumar and Manoharan, 1996). Determination of dry matter content was done gravimetrically (Sathishkumar and Manoharan, 1996) and that of phospholipids and galactolipids was accomplished through lipid phosphorus (Wagner et al., 1962) and lipid galactose (Roughan and Batt, 1968), respectively. Total leaf-chlorophyll content was estimated according to the procedure of Arnon (1949).

Analysis of nDNA Fragmentation

nDNA was isolated by CTAB method according to Rogers and Bendich (1994) and electrophoresced in 0.75% agarose gel according to Sambrook et al. (1989) and Mittler and Lam (1995).

Catalase Activity

Catalase activity was determined according to Aebi (1984). Enzyme extract was prepared from 500 mg fr wt of leaves in 1.5 mL of 1% Triton X-100 prepared in 50 mM phosphate buffer, pH 7.0. To 200 μL of clear homogenate, 1.8 mL of phosphate buffer, pH 7.0, was added. Reaction was started with the addition of 1 mL of 30 mM H_2O_2 prepared in phosphate buffer, pH 7.0. Absorbance was monitored at 240 nm during the reaction. Catalase activity was expressed as nmol H_2O_2 scavenged $min^{-1}mg^{-1}$ protein. Protein estimation was done according to Lowry et al. (1951).

Data Presentation

Results are the mean of three independently carried out experiments. Sampling for each determination was from a minimum of randomly picked up 20 seedlings. The SD for different parameters are as follows: dry-matter content, ca. 6%; % composition of individual phospholipids and galactolipids, ca. 3%; total protein content, ca. 2%. Data presented are the mean ± SD.

RESULTS AND DISCUSSION

Triple Response Phenotype and the Characterization of 2,4-D-Induced Leaf Senescence and Senescence Arrest by $AgNO_3$ in Mustard Seedlings

Ecker (1995) reported the phenotypic characteris-

tics in relation to the operation of ethylene-mediated signal transduction pathway in pea seedlings. Growth aberrations such as inhibition of epicotyl and root elongation, radial swelling of epicotyl and root cells and the development of a horizontal growth habit were observed in response to ethylene treatment. These aberrations were collectively termed as "triple response phenotype". In the present study, mustard seedlings exhibited the triple response phenotype, which revealed the occurrence of hormonal cross talk involving endogenous ethylene during 2,4-D-induced leaf senescence. Results showed that the employed morphometric parameters along with total chlorophyll content were sensitive and useful in evaluating the triple response characteristics associated with different experimental conditions of the present study

Table 2. Changes in the phospholipid and galactolipid composition due to 2,4-D-induced senescence and senescence-arrest by 2,4-D+$AgNO_3$ supplementation in mustard leaves: 2,4-D (500 μM) and 2,4-D (500 μM)+$AgNO_3$ (0.1 mM) were supplemented to 4 d old mustard seedlings. Analysis of membrane lipids was carried out in leaves collected 4 d subsequent to supplementation of experimental solutions.

Treatment	Phospholipids + galactolipids (μmol g^{-1} dry wt)	Phospholipids + galactolipids (% total)										
		PC	LPC	PE	LPE	PI	PS	PG	PA	MGDG	DGDG	SL
Control-H_2O	33.5 ± 1.90	27	nd	19	Nd	10	06	04	02	20	09	03
Control-0.1% acetone	32.9 ± 1.65	28	nd	19	Nd	10	06	05	02	19	08	03
2,4-D	31.0 ± 1.50	09	17	13	05	08	06	02	14	15	09	02
2,4-D + $AgNO_3$	33.2 ± 1.54	26	02	18	01	08	06	04	06	18	08	03

nd, not detected.

Table 3. Changes in the content of lipolytic products of phospholipids due to 2,4-D-induced senescence and senescence-arrest by 2,4-D+$AgNO_3$ supplementation in mustard leaves: 2,4-D (500 μM) and 2,4-D (500 μM)+$AgNO_3$ (0.1 mM) were supplemented to 4 d old mustard seedlings. Analysis of phospholipids was carried out in leaves collected at specific time points subsequent to supplementation of experimental solutions.

Treatment	Day(s) after treatment	Phopholipid content (μmol g^{-1} dry wt)		
		LPC	LPE	PA
Control-H_2O	0	nd	nd	0.63 ± 0.009
	1	nd	nd	0.60 ± 0.011
	2	nd	nd	0.63 ± 0.014
	3	nd	nd	0.59 ± 0.007
	4	nd	nd	0.67 ± 0.011
Control-0.1% acetone	0	nd	nd	0.63 ± 0.013
	1	nd	nd	0.61 ± 0.012
	2	nd	nd	0.64 ± 0.016
	3	nd	nd	0.61 ± 0.009
	4	nd	nd	0.66 ± 0.013
2,4-D	0	nd	nd	0.63 ± 0.013
	1	nd	nd	0.60 ± 0.008
	2	1.42 ± 0.018	0.09 ± 0.002	1.56 ± 0.027
	3	3.32 ± 0.060	0.70 ± 0.018	3.12 ± 0.054
	4	5.27 ± 0.090	1.55 ± 0.023	4.34 ± 0.081
2,4-D + $AgNO_3$	0	nd	nd	0.63 ± 0.010
	1	nd	nd	0.72 ± 0.016
	2	0.36 ± 0.0063	nd	0.78 ± 0.023
	3	0.59 ± 0.009	0.28 ± 0.006	0.73 ± 0.014
	4	0.66 ± 0.009	0.33 ± 0.007	1.99 ± 0.032

nd, not detected.

(Table 1). Especially, shoot weight : specific shoot growth ratio significantly marked leaf senescence due to 2,4-D supplementation and also senescence arrest by 2,4-D+$AgNO_3$. Seedlings supplemented with 500 μM 2,4-D showed decrease in the shoot weight : specific shoot growth ratio and total chlorophyll content, by ca. 30% and 57% respectively on day 4, as compared to acetone control. Gepstein et al. (2003) employed senescing leaves that had 60% of total chlorophyll content as compared to control leaves, in their study on the identification of senescence associated genes (SAG) in *A. thaliana*. There was only a marginal increase in shoot length and seedling length, by ca. 16% and 14%, respectively, due to 2,4-D supplementation at 300 μM, which possibly indicated weak growth stimulation activity of 2,4-D at this concentration. 2,4-D beyond 400 μM was growth inhibitory and senescence inducing wherein changes in morphometric parameters and total chlorophyll content were more pronounced at 500 and 600 μM of 2,4-D. Thus, there was a transition observed in 2,4-D action wherein 2,4-D acted both as an elongation factor as well as a senescence-factor in a concentration dependant manner. Decrease in total chlorophyll content was much the same by ca. 57% due to 2,4-D supplementation at 500 and 600 μM. There was no significant effect on the specific growth of shoot and root in all the experimental treatments of the present study.

Subsequent to the preparative evaluation of mustard seedlings on the basis of "triple response phenotype", subsequent experiments were carried out employing a set of biochemical and molecular parameters in characterizing 2,4-D-induced leaf senescence. Observations showed conclusively that the different parameters of the present study were insensitive to 0.1% acetone, which were otherwise sensitive to the employed experimental conditions (Table 1, 2, 3, 4; Fig. 1).

Table 4. Changes in catalase activity during to 2,4-D-induced senescence and senescence-arrest by 2,4-D+$AgNO_3$ supplementation in mustard leaves: 2,4-D (500 μM) and 2,4-D (500 μM)+$AgNO_3$ (0.1 mM) were supplemented to 4 d old mustard seedlings. Catalase activity was determined in leaves collected at specified time points subsequent to supplementation of experimental solutions.

Treatment	Day(s) after treatment	Enzyme activity (nmol H_2O_2 scavenged min^{-1} mg^{-1} protein)
Control-H_2O	0	0.05 ± 0.001
	1	0.06 ± 0.002
	2	0.06 ± 0.001
	3	0.05 ± 0.002
	4	0.05 ± 0.002
Control-0.1% acetone	0	0.06 ± 0.002
	1	0.05 ± 0.002
	2	0.06 ± 0.002
	3	0.06 ± 0.001
	4	0.05 ± 0.002
2,4-D	0	0.06 ± 0.001
	1	0.12 ± 0.004
	2	0.16 ± 0.006
	3	0.16 ± 0.005
	4	0.09 ± 0.003
2,4-D + $AgNO_3$	0	0.06 ± 0.002
	1	0.09 ± 0.002
	2	0.08 ± 0.003
	3	0.06 ± 0.001
	4	0.06 ± 0.002

2,4-D-Induced Leaf Senescence and Hormonal Interactions

The present study was focused on the characterization of early and terminal phases of leaf-senescence due to 2,4-D supplementation. Terminal phase of leaf senescence is considered to represent a point of no return from the progression and subsequent completion of the senescence pathway (Pennell and Lamb, 1997). The present study has relevance to the parallel work being carried out in our laboratory on the characterization of PCD in relation to induction of somatic embryogenesis in legume tissue cultures. These studies are focused on the xylogenesis related PCD and the formation of somatic embryos wherein PCD markers are evaluated for the embryogenic potential of cultured cells.

In the present study, induction of leaf senescence was observed due to supplementation of 2,4-D at 500 μM to 4-day old seedlings (Table 1). Among the tested concentrations of 2,4-D, in the range of 300 to 600 μM, 500 μM was found to be optimal to induce leaf senescence and also to attain terminal phase of senescence on day 4 (Table 1, Fig. 1). IAA at equimolar concentration was comparatively less effective than 2,4-D in inducing leaf senescence (Table 1). Differential effect of IAA and 2,4-D could be attributed to differences in their uptake, formation of conjugated forms, degradation by cellular auxin degrading enzymes and the consequent accumulation of these

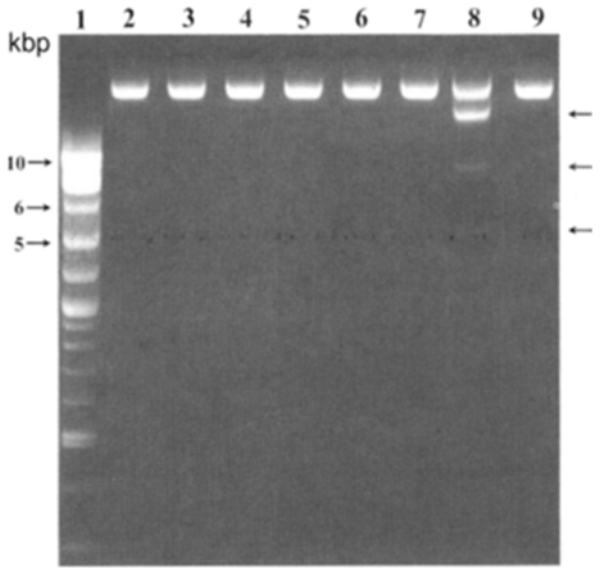

Figure 1. nDNA profile of leaves during 2,4-D-induced senescence in mustard. 2,4-D (500 μM) and 2,4-D (500 μM)+$AgNO_3$ (0.1 mM) were supplemented to 4 d old mustard seedlings. nDNA was characterized in leaves collected at specified time points subsequent to supplementation of experimental solutions. Age of plants subsequent to the supplementation is designated as 0 d, 1 d, 2 d, 3 d, and 4 d. Lane 1, 1 kb ladder marker DNA; lane 2, 0 d 2,4-D; lane 3, 1 d 2,4-D; lane 4, 2 d 2,4-D; lane 5, 3 d 2,4-D; lane 6, 4 d H_2O control; lane 7, 4 d 0.1% acetone control; lane 8, 4 d 2,4-D; lane 9, 4 d 2,4-D + $AgNO_3$.

native and synthetic auxins, respectively (Sembdner et al., 1994). In the present study, experiments were also carried out to find out the effect of Ag^+ ions, an inhibitor of ethylene biosynthesis, on the 2,4-D-induced senescence (Table 1; Hansen and Grossmann, 2000). Ag^+ ions, supplemented in the form of $AgNO_3$, inhibited 2,4-D-induced leaf senescence. Among the tested concentrations of $AgNO_3$, in the range of 0.05 to 0.25 mM, 0.1 mM was found to be effective in antagonizing 2,4-D-induced leaf senescence. It is pertinent to mention that $AgNO_3$ beyond 0.25 mM got adsorbed to the cotton-pad substratum on which seedlings were grown and that formed a mat like appearance.

Auxin-induced senescence at supra-optimal concentrations has been shown to occur at external concentration of 100 μM and 500 μM IAA in 3 weeks old hydroponically grown seedlings of *Galium aparine* and tomato, respectively (Hansen and Grossmann, 2000). In the present study, optimization of irrigation volume of experimental solutions in relation to the age of seedlings as well as the intactness of roots was carried out on the basis of morphological appearance. These preparative aspects were carried out in order to characterize senescence without possible interference by exudates that were formed due to root damage especially under prolonged incubations. It was observed that seedlings when grown beyond day 4 in 2,4-D and 2,4-D+$AgNO_3$ exhibited horizontal growth due to progression of senescence. It is relevant to mention that the horizontal growth of seedlings constituted one of the "triple response phenotype" in response to ethylene, which is possibly an intermediary signal in the 2,4-D-induced leaf senescence (Ecker, 1995). Horizontal growth of seedlings lead to shoots coming in direct contact with experimental solutions, which resulted in hydration and softening of shoots. Subsequently seedlings in Petri dish got contaminated, which made the system unsuitable beyond day 4. Hence, experiments were limited to day 4.

Senescence Associated Changes in Membrane Lipid Composition, Degradation of Membrane Lipids, and Accumulation of Lipolytic Products of Phospholipids

A set of experiments were undertaken to find out the fate of membrane lipids in response to 2,4-D supplementation. It is known that leaf senescence results in degradation and disassembly of endomembrane systems (Thomas and Stoddart, 1980; Pennell and Lamb, 1997). Experiments focused on the qualitative and quantitative composition of phospholipids and galactolipids during 2,4-D-induced leaf senescence and senescence arrest by $AgNO_3$ (Table 2, 3). Observations showed that there was no significant change in the total content of phospholipids+galactolipids either due to 2,4-D or 2,4-D+$AgNO_3$ supplementation as compared to control (Table 2). The leaves from control seedlings had the following qualitative composition of membrane lipids, in the following order of their predominance: phosphatidylcholine (PC)> phosphatidylethanolamine (PE)>phosphatidylinositol (PI) > phosphatidylserine (PS)>phosphatidylglycerol (PG) > phosphatidic acid (PA) among phospholipids and monogalactosyl diglyceride (MGDG) > digalactosyl diglyceride (DGDG) > sulpholipid (SL) among galactolipids (Table 2). Qualitative composition of membrane lipids of mustard leaves is comparable to that of rice callus cultures that was reported from our laboratory (Sathishkumar and Manoharan, 1996). In addition to the phospholipid and galactolipid species of the control, senescing leaves contained lyso-derivatives of PC and PE, namely, LPC and LPE. Even though LPC and LPE

were present in 2,4-D+$AgNO_3$ supplemented samples, their relative level was very low as compared to 2,4-D supplemented samples (Table 2). The content of the lytic products phosphlipids was in the following order of their predominance on day 4 due to 2,4-D supplementation: LPC>PA>LPE (Table 3). It has been shown that activity of lipid-hydrolyzing enzymes was induced by several abiotic stress factors such as osmotic-, salinity- and cold-stress (Zhu, 2002). Observations of the present study showed that 2,4-D supplementation leads to significant decrease in the relative level of PC and PE on day 4 by ca. 68% and 32%, respectively (Table 2). Accumulation of LPC and LPE could be observed from day 2 onwards in senescing leaves (Table 3). There was also accumulation of PA, a PLD product, in this system. Relative level of PA increased 7 fold due to 2,4-D supplementation as compared to control. Increase in the relative level of PA was also reflected in the content of PA, which showed ca. 6.9 fold increase on day 4 due to 2,4-D supplementation as compared to its respective control (Table 3). Increase in PA content due to 2,4-D+$AgNO_3$ supplementation was only ca. 3 fold which was significantly lower as compared to 2,4-D supplementation. Activation of a family of PLD contributed to the degradation of cellular phospholipids leading to accumulation of PA in a few systems that were subjected to environmental stress (Chapman, 1998; Wang, 2000, 2001; Zhu, 2002). Based on the genomic level differential expression analysis during senescence, it has been shown that PLD is a differentially expressed gene product that was upregulated (Bhalerao et al., 2003; Gepstein et al., 2003). Accordingly, PLD has been termed as a senescence-associated gene (SAG) in *A. thaliana* and *Populus tremula*. Besides the change in the relative level of major phospholipids, there was significant decrease in the relative level of minor phospholipids, namely PI and PG, due to 2,4-D supplementation. Whereas decrease in the relative level of PI was marginal, that of PG, a specific phospholipid of chloroplast showed decrease by ca. 60% (Table 2; Harwood, 1980). Relative level of PS was insensitive to 2,4-D supplementation. Thus, the results showed that senescing leaves of mustard seedlings possibly operated PLD-mediated phospholipid degradation during 2,4-D-induced leaf senescence. Anti-sense suppression of PLD-Dα and the resulting inhibition of ethylene- and ABA-mediated leaf senescence have been reported in *A. thaliana* (Fan et al., 1997). Formation of LPC and LPE due to 2,4-D supplementation possibly indicates the involvement of PC- and PE-fatty acyl hydrolase(s) during 2,4-D-induced leaf senescence in mustard seedlings. However, these gene products are not found among the annotated senescence associated genes or gene products in *A. thaliana* and *P. tremula* (Bhalerao et al., 2003; Gepstein et al., 2003). Further work is needed to confirm these findings of the present study. In the present study there was senescence arrest together with significant changes in the relative level of phospholipids and galactolipids as well as the content of LPC, LPE and PA due to 2,4-D+$AgNO_3$ supplementation. These changes corresponded to reversal of changes in these parameters that was brought about by 2,4-D when supplemented alone. However, $AgNO_3$-mediated senescence arrest and the associated reversal in lipid profile were not total as revealed in the content of LPC, LPE and PA (Table 3).

It is known that during senescence different subcellular compartments underwent disassembly in a defined time course (Thomas and Stoddart, 1980; Quirino et al., 2000). Chloroplasts degraded during early phase of leaf senescence (Harwood, 1980; Greenberg, 1996; Sathishkumar and Manoharan, 1996; Quirino et al., 2000). Results of the present study showed that the relative level of MGDG showed decrease by ca. 21% on day 4 due to 2,4-D supplementation (Table 2). Relative level of DGDG and SL did not undergo significant change and thus remained insensitive during 2,4-D-induced leaf senescence. It is known that galactolipids constitute chloroplast-specific membrane lipids and MGDG and DGDG are specifically localized in the grana and envelope factions of chloroplasts, respectively (Harwood, 1980; Quirino et al., 2000). Accordingly, MGDG:DGDG ratio has been found to be a marker in the evaluation of intactness or degraded status of chloroplast membrane (Harwood, 1980; Sathishkumar and Manoharan, 1996). MGDG:DGDG ratios of leaves were 2.4 and 1.7 in the control and 2,4-D treated samples, respectively (Table 2). Decrease in MGDG:DGDG ratio due to 2,4-D supplementation revealed the degradation of grana. Contrastingly, MGDG:DGDG ratio of 2,4-D+$AgNO_3$ samples was comparable to that of the control which reflected intactness of grana and also senescence arrest by $AgNO_3$. Concomitant with the degradation of grana, there was decrease in the total chlorophyll content in the senescing leaves (Table 1). Whereas there was ca. 57% reduction in the total chlorophyll content on day 4 in 2,4-D treated samples, 2,4-D+$AgNO_3$ treated samples as well as control had comparable amount of chlorophylls. It is pertinent to mention that on day 4, the leaves showed wilting symptoms without under-

going further degradation of chlorophylls and consequently there was only slight yellowing of the leaves in mustard seedlings. Gepstein et al. (2003) employed leaves that showed 60% decrease in total chlorophyll content as compared to control for the identification of SAG in *A. thaliana*. System of the present study is comparable to the senescing system of *A. thaliana*, on the basis of total chlorophyll content (Table 1; Gepstein et al., 2003). Changes in the composition of leaf pigments and an associated pattern of color changes during senescence have been reported in autumn leaves of aspen (Bhalerao et al., 2003).

Stimulation of Catalase Activity during Early Phase of Senescence

Senescence is a genetically regulated oxidative process during which strong enhancement in the generation of ROS has been reported (Pellinen et al., 2002; Dat et al., 2003). It is known that expression of ROS-scavenging gene products such as catalases, peroxidases and superoxide dismutases is upregulated during senescence in order to detoxify ROS (Thomas and Stoddart, 1980; Bhalerao et al., 2003; Gepstein et al., 2003; Overmeyer et al., 2003). In the present study, a set of experiments was undertaken to determine the activity of catalase (EC 1.11.1.6) in order to assess ROS-scavenging activity during 2,4-D-induced leaf senescence. Results showed that catalase activity was at its peak on day 3, which was 2.7 fold higher due to 2,4-D supplementation as compared to its respective control (Table 4). 2,4-D+$AgNO_3$ supplementation resulted in significant decrease in catalase activity as compared to 2,4-D when supplemented alone. This possibly indicates decreased ROS production and an associated decrease in catalase activity due to $AgNO_3$-mediated senescence inhibition. Expression of ROS-scavenging gene products has been shown to be a characteristic feature of systems that undergo senescence or PCD (Pennell and Lamb, 1997; Bhalerao et al., 2003; Gepstein et al., 2003). Activity profile of catalase has been shown to serve as an indicator of cellular level detoxification activity, especially under stress conditions (Thomas and Stoddart, 1980; Overmeyer et al., 2003). Accordingly, stimulation of catalase activity due to 2,4-D and its inhibition due to supplementation of 2,4-D+$AgNO_3$ in the present study revealed the role of this marker enzyme in ROS scavenging during the early phase of senescence. However, prolongation of senescence inducing conditions is known to result in the progression of senescence due to cessation of ROS scavenging activity (Thomas and Stoddart, 1980; Pellinen et al., 2002).

Genomic DNA Degradation Marks the Terminal Phase of Senescence

Hypersensitive response (HSR), a form of PCD, is known to share common biochemical and molecular features with leaf senescence (Thomas and Stoddart, 1980; Ryerson and Heath, 1996; Orzaez and Granell, 1997). HSR has been shown to be associated with the induction of endonucleases activity due to viral pathogenesis in tobacco leaves (Mittler and Lam, 1995). Degradation of nDNA has been implicated as a death signal that was associated with PCD in this system. Occurrence of nDNA fragmentation marked the terminal phase of senescence, which was considered to represent a point of no return in the PCD pathway during pathogenesis (Greenberg, 1996; Pennell and Lamb, 1997). Gepstein et al. (2003) reported endonuclease gene as one of the SAG in *A. thaliana*. Cleavage of nDNA into oligonucleosomal fragments has also been shown to occur during cell death due to fungal infection or abiotic stress (Ryerson and Heath, 1996). In the present study, a set of experiments was undertaken to mark the terminal phase of 2,4-D-induced leaf senescence of mustard seedlings on the basis of nDNA degradation. Accordingly, kinetic analysis of nDNA profiles of leaf tissue at 24 h intervals was carried out subsequent to 2,4-D and 2,4-D+$AgNO_3$ supplementation. Observations showed that nDNA was intact and did not undergo fragmentation till day 3. This was visualized by the appearance of genomic DNA as a crisp band without any detectable fragment in agarose gels (Fig. 1, lanes 2-5). Contrastingly, nDNA from day 4 samples showed a few low molecular weight fragments that were formed due to 2,4-D-induced leaf senescence (Fig. 1, lane 8). Though there was reproducible level of nDNA fragmentation in day 4 leaves, significant amount of nDNA still remained intact without undergoing nucleolytic attack. Since the objective of the present study was to mark the terminal phase of senescence, these observations were considered significant in marking the "point of no return" in the 2,4-D-induced leaf senescence pathway.

Efficacy of Ag^+ ions in the inhibition of 2,4-D-induced leaf senescence and the associated inhibition of nDNA degradation was also significantly visualized in agarose gels of 2,4-D+$AgNO_3$ treated samples. It is known that there exists a family of genes coding for nucleases in tobacco, termed as NUCI, NUCII, and NUCIII (Mittler and Lam, 1995). Thus, nDNA profile

pattern due to 2,4-D-induced leaf senescence of mustard seedlings is comparable to the HSR in tobacco (Mittler and Lam, 1995). Also, the results of the present study showed the occurrence of membrane damage prior to nDNA degradation, wherein these events marked the early- and late-phases of senescence (Table 2, 3; Fig. 1).

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