CHAPTER IV

INTERACTION OF PROLINE WITH ABSCISIC ACID (ABA) AND Ca^{2+} ANTAGONISTS IN AFFECTING THE SENESCENCE OF EXCISED LEAVES
Abscisic acid-Proline interaction in acceleration of senescence of excised leaves

The leaf senescence accelerating role of abscisic acid (ABA) is well established and documented (e.g., Zeevaart and Creelman, 1988). Enhanced ABA contents of leaves constitute, among other factors, a crucial component of regulation of senescence process. Besides, ABA is known to accumulate in plants during several abiotic stresses e.g., water deficit, salinity etc. Since proline also accumulates in plant tissues both during abiotic stresses and senescence, it was of interest to examine the interaction, if any, between ABA and proline, which co-occur in the senescing leaves in affecting the senescence of excised leaves. The interactive effects of L-Pro and ABA were expected to provide insight into the regulation of leaf senescence by L-Pro. Furthermore, one of the objectives of present study was to examine the dependence of L-Pro, if any, on Ca\(^{2+}\) availability for imposing its effects. Since ABA is already established to rely on Ca\(^{2+}\) ions for its action, ABA was involved in these studies.

For these interaction experiments, the physiologically relevant concentrations of both effectors were employed; thus, ABA and proline were used in micro- and millimolar concentration range, respectively. Further, in view of the strong senescence accelerating effects of ABA and also (although not comparable to ABA) those of L-Pro, the interaction was studied under illumination through a different experimental design. The latter involved infiltration of excised leaves with proline solutions of desired concentrations through petiole in glass vials. Following 3 h of starting
proline infiltration, ABA was applied to both leaf surfaces. Leaves were allowed to senesce under illumination. The latter induced a slower senescence as compared to the dark-induced one.

**Effect of exogenous ABA on excised leaf senescence**

Prior to examining the interaction between ABA and proline, the individual effects of ABA on leaf senescence were characterized by monitoring the senescence parameters in leaves floating on ABA solution in dark. As expected, exogenous ABA induced a strong and concentration dependent acceleration of senescence of excised *T. majus* leaves as revealed in chlorophyll loss. At a concentration of ABA as low as 0.1 μM, leaf chlorophyll contents were already reduced by 27% as compared to the control. An increase in ABA concentration intensified the senescence acceleration; at 100 μM ABA, chlorophyll contents were only 23% those of control leaves (Fig. 4.1A, 4.2). Chl a : Chl b ratio of senescing leaves was reduced marginally until 10 μM ABA but at 100 μM ABA, the ratio was drastically lowered. The Chl a : b ratio was 87 and 52% of control values, respectively at 10 and 100 μM ABA (Fig. 4.1B). These data indicate enhanced sensitivity of Chl a to higher ABA concentrations applied.

The senescence dependent proline accumulation in leaves was further enhanced by exogenous ABA; the ABA effect was not much until 1 μM concentration. Thus, free proline contents of leaves senescing with 1 and 100 μM ABA were 110 and 140% of control senescing leaves, respectively (Fig. 4.3A). The Lipid peroxidation (MDA contents) and K⁺ leakage from senescing leaves were also promoted by ABA in a
Fig. 4.1. Effect of exogenous abscisic acid (ABA) on total chlorophyll contents (A) and Chl a/b ratio (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. $n = 9 \pm s.d.$

Initial (0 d) values:
Total chlorophyll: $3.41 \pm 0.02$ mg g$^{-1}$ FW
Chl a/b ratio: $3.00 \pm 0.25$
Fig. 4.2. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous abscisic acid (ABA). Dark incubated (5 d) leaves with different ABA concentrations.
Fig. 4.3. Effect of exogenous abscisic acid (ABA) on free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. *n* = 9 ± s.d.

Initial (0 d) values:
Free proline: 0.22 ± 0.03 μmol g⁻¹ FW
MDA: 0.084 ± 0.012 μmol g⁻¹ FW
K⁺ leakage: Not detected
concentration dependent manner. The effect of ABA on K⁺ leakage was more prominent as compared to that on lipid peroxidation. For example, ABA (100 μM) induced an increase of 44% in MDA contents of senescing leaves which was accompanied by a rise of 11.5 fold in K⁺ leakage into the incubation medium (Fig. 4.3B, C).

ABA is a stronger inducer of stomatal closure (e.g., Mansfield et al., 1990). Accordingly, in these experiments also, ABA-induced stomatal closure was revealed in enhanced SDR and reduced rate of transpiration. ABA-induced increase in SDR was evident even at 0.1 μM ABA where SDR was already 2.4- fold of that in control leaves. With a further increase in ABA concentration, SDR increased linearly leading to a 6.3- fold increased SDR at 100 μM ABA (Fig. 4.4A). The rate of transpiration was invariably related inversely to the SDR values. Thus, the transpiration rate at 0.1 and 10 μM ABA were 75 and 22% of those in control leaves, respectively (Fig. 4.4B).

**Interaction between proline and ABA in affecting the leaf senescence**

Leaf infiltration with proline through petiole in *T. majus* did not affect the chlorophyll loss induced by dark incubation at lower concentrations (0.1, 1.0 mM). However, at 10 mM proline chlorophyll loss of senescing leaves was enhanced; these leaves contained 80% chlorophyll that of control. Apparently, the proline-induced acceleration of senescence in this experimental system (petiole infiltration under illumination) was of much lower magnitude when compared to that occurring in leaves floating in dark (described in previous chapter; Fig. 3.8A). The delayed leaf senescence in
Fig. 4.4. Effect of exogenous abscisic acid (ABA) on stomatal diffusive resistance (SDR) (A) and rate of transpiration (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. n = 9 ± s.d.
the former could be ascribed to the illumination conditions. ABA (0.1 mM), when applied individually to the leaf surfaces induced a chlorophyll loss; ABA treated leaves contained 82% chlorophyll as compared to the control. Once again, the ABA effects (Fig. 4.5, 4.6) under illumination were lower in magnitude as compared to those occurring in darkness as described earlier (Fig. 4.1, 4.2). Concerning the interactive effects of ABA and proline, ABA-induced promotion of chlorophyll loss was not much affected by a simultaneous application of equimolar (0.1 mM) Pro. But at higher proline levels (1, 10 mM), the ABA dependent promotion of chlorophyll loss was further enhanced. Thus, leaves treated with ABA alone contained 82% chlorophyll that of control; these values were reduced to 67 and 61% of control, respectively by a simultaneous application of 1 and 10 mM proline (Fig. 4.5A; Fig. 4.6). Chl a : b ratio of senescing leaves was lowered due to infiltration with lower concentrations of proline (0.1, 1.0 mM); at 10 mM proline the ratio recovered to the control levels. Leaf infiltration with ABA (0.1 mM) individually resulted in lowered Chl a : Chl b ratio. This reduction was reverted by a simultaneous application of proline particularly 10 mM (Fig. 4.5B).

The endogenous leaf proline contents of senescing leaves (petiole infiltrated) with exogenous proline did not change much until 1.0 mM concentration. Treatment with 10 mM proline, however, resulted in 6.8-fold increased endogenous proline levels as compared to control (Fig. 4.7A). ABA, applied individually also caused enhancement of endogenous proline (234% of control). A simultaneous application of proline induced a large and concentration dependent increase in endogenous proline contents. For
Fig. 4.5. Total chlorophyll contents (A) and Chl a/b (B) of senescing excised leaves of *Tropaeolum majus* under illumination as affected by exogenous proline (L-Pro) and ABA, applied individually or in combination. ABA was applied to the leaf surfaces whereas proline was supplied through petiole.

Data after 5 d of dark incubation. n = 6 ± s.d.

Initial (0 d) values:
Total chlorophyll: 3.25 ± 0.07 mg g⁻¹ FW
Chl a/b ratio: 3.12 ± 0.02
Fig. 4.6. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous proline (L-Pro) and abscisic acid (ABA), applied individually or in combination. Leaves treated with L-Pro (petiole infiltration) and/or ABA applied to both surfaces for 5 d under illumination.
example, leaves treated with 0.1 mM ABA+10 mM proline contained 13.8-fold greater endogenous proline as compared to leaves treated with 0.1 mM ABA alone (Fig. 4.7A).

Lipid peroxidation (MDA contents) of proline infiltrated senescing leaves was only slightly enhanced as compared to that of control. ABA (0.1 mM) individually also induced a marginal increase in MDA contents, which was further increased by a simultaneous application of exogenous proline (Fig. 4.7B). As was the case with MDA contents, exogenous proline induced the promotion of K⁺ leakage from senescing leaves particularly at 10 mM concentration. A 2.7-fold increased K⁺ leakage with 10 mM proline treatment was observed. ABA (0.1 mM) caused a 3.7-fold increase in K⁺ leakage as compared to control. This was strongly enhanced, in a concentration dependent manner, by the simultaneous application of proline. For example, K⁺ leakage from the senescing leaves with 0.1 mM ABA + 1 mM proline was 2.8-fold that from the leaves senescing with ABA (0.1 mM) alone (Fig. 4.7C).

The treatment of senescing leaves with exogenous proline resulted in enhanced stomatal diffusive resistance implying the closure of stomata. The SDR of leaves treated with 10 mM proline was 16-fold that of control leaves. ABA (0.1 mM), applied alone, also caused an enhancement of SDR. This was further enhanced by a simultaneous application of proline. For example, SDR of the leaves treated with 0.1 mM ABA + 10 mM proline was 4.7-fold as compared to the leaves treated with 0.1 mM ABA alone. SDR was inversely related with the rates of transpiration of leaves treated with different effectors, either individually, or in combination (Figs. 4.8A, B).
Fig. 4.7. Free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* under illumination as affected by exogenous proline (L-Pro) and ABA, applied individually or in combination. ABA was applied to the leaf surfaces whereas proline was supplied through petiole.

Data after 5 d of dark incubation. n = 6 ± s.d.

Initial (0 d) values:
Free proline: $0.02 \pm 0.03 \mu$mol g⁻¹ FW
MDA: $0.078 \pm 0.05 \mu$mol g⁻¹ FW
K⁺ leakage: Not detected
Fig. 4.8. Stomatal diffusive resistance (SDR) (A) and rate of transpiration (TR) (B) of senescing excised leaves of *Tropaeolum majus* under illumination as affected by exogenous proline (L-Pro) and ABA, applied individually or in combination. ABA was applied to the leaf surfaces whereas proline was supplied through petiole.

Data after 5 d of dark incubation. n = 6 ± s.d.
Interaction between proline and Ca\(^{2+}\) antagonists in affecting the leaf senescence

Ca\(^{2+}\) regulates an array of plant physiological processes including leaf senescence e.g., by stabilizing the membrane systems. Among other functions, Ca\(^{2+}\) has been implicated in coordination of the effects of diverse phytohormones e.g., those of ABA (Mansfield et al., 1990; Sharma et al., 1995) and kinetin (Nooden and Leopold, 1980). Assuming a similarity between ABA and proline-induced accumulation of leaf senescence, it was considered to be of interest to examine the requirement of Ca\(^{2+}\), if any, for exogenous proline-dependent acceleration of senescence of excised *T. majus* leaves in darkness. For this objective, the proline-induced senescence was monitored in the presence of certain Ca\(^{2+}\) chelators (EDTA, EGTA) and Ca\(^{2+}\) channel blockers (nifedipine, verapamil). These substances were applied with an idea of manipulating the endogenous Ca\(^{2+}\) levels such that proline effects could be studied at lowered cellular Ca\(^{2+}\) levels.

**Interaction between proline and Ca\(^{2+}\) chelators (EDTA, EGTA)**

With the application of ethylenediamine tetraacetic acid (EDTA) to excised *T. majus* leaves, chlorophyll loss was promoted at 1 and 10 mM concentration. However, at 100 mM EDTA, chlorophyll contents were higher than those in control (Fig. 4.10A, 4.11). EDTA (100 mM) treated leaves appeared peculiar in that they had become very thin and fragile but retained the chlorophyll contents. As described earlier, 10 mM Pro also accelerated the rate of chlorophyll loss; leaves contained 56% chlorophyll that of control at this concentration. The proline dependent enhancement of
Fig. 4.10. Effect of exogenous ethylenediamine tetraacetic acid (EDTA) and proline (L-Pro) applied, alone and simultaneously on total chlorophyll contents (A) and Chl a/b ratio (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. n = 9 ± s.d.

Initial (0 d) values:
- Total chlorophyll: 2.96 ± 0.22 mg g⁻¹ FW
- Chl a/b ratio: 2.88 ± 0.39

* Fragile and very thin leaves
chlorophyll loss was reversed in the presence (simultaneous) of EDTA. For example, leaves at 10 mM proline+10 mM EDTA contained 2-fold chlorophyll that of the leaves treated with proline alone (Fig. 4.10A, 4.11). Likewise, the Chl a : Chl b ratio was marginally lowered due to 10 mM Pro. This reduction was also reverted to control values by a simultaneous application of EDTA (Fig. 4.10B).

The free proline contents of senescing leaves, were not affected much by 1 and 10 mM EDTA. However, these were reduced to 24% of control at 100 mM EDTA. Treatment with exogenous proline led to the enhanced endogenous proline contents. A simultaneous treatment with proline and EDTA caused a general increase in endogenous proline levels; the concentration-dependence, however, was not clear. For example, proline (10 mM) treated leaves contained 170% endogenous free proline that of control whereas this value was increased to 347% of control with a simultaneous application of 1 mM EDTA (Fig. 4.12A).

The MDA contents of senescing leaves were differentially affected by EDTA, these were slightly promoted by lower (1, 10 mM) and strongly inhibited by higher (100 mM) EDTA concentration. Irrespective of their individual effects, however, all EDTA concentrations reversed the exogenous proline-induced increase in MDA contents. For example, 10 mM proline treated leaves contained 133% MDA that of control leaves which was reduced to 60% of control in the presence of 10 mM EDTA (Fig. 4.12B). Showing a correspondence with MDA contents, with an individual application of EDTA, K⁺ leakage increased. Proline induced K⁺ leakage was further enhanced with a simultaneous application of EDTA (Fig. 4.12C).
Fig. 4.11. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous ethylenediamine tetraacetic acid (EDTA) and proline (L-Pro), applied individually or in combination. Leaves treated with EDTA and/or L-pro for 5 d in darkness.
Fig. 4.12. Effect of exogenous ethylenediamine tetraacetic acid (EDTA) and proline (L-Pro) applied, alone and simultaneously, on free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. n = 9 ± s.d.

Initial (0 d) values:
Free proline: 0.21 ± 0.02 μmol g⁻¹ FW
MDA: 0.072 ± 0.01 μmol g⁻¹ FW
K⁺ leakage: Not detected
* Not detected
The influence of EGTA, applied alone or in combination with proline, on senescence of excised leaves generally resembled that of EDTA in pattern but differed in magnitude. Thus, EGTA caused a slight promotion of chlorophyll loss at 1 and 10 mM. Leaves treated with 100 mM EGTA differed altogether; after 5 d dark incubation they were quite green and retained more than control levels of chlorophylls although they had become unusually thin. Exogenous proline-induced promotion of chlorophyll loss was not changed by lower EGTA concentrations (1, 10 mM); only a marginal reversal of loss was observed with 1 mM EGTA. Leaves treated with 10 mM proline + 100 mM EGTA showed substantially more chlorophyll levels as compared to the proline treated leaves (Fig. 4.13A; 4.14). Chlorophyll a: Chlorophyll b ratio of senescing leaves was not altered much by EGTA. A slight reduction in the ratio due to 10 mM proline was reversed to the control values by simultaneous application of EGTA (Fig. 4.13B).

Free proline contents of the senescing leaves were suppressed by EGTA (≥10 mM); 100 mM EGTA-treated leaves contained 15% proline levels those of control. The proline treatment dependent increase in endogenous proline was further enhanced by 1 mM EGTA but strongly lowered at EGTA concentrations more than 10 mM (Fig. 4.15A). The MDA contents of senescing leaves were not affected by lower concentration of EGTA (1 mM), but were lowered by the higher ones (10, 100 mM). Proline induced increase in MDA contents was suppressed by EGTA in a concentration dependent manner (Fig. 4.15B). Although MDA contents were slightly lowered by EGTA, K⁺ leakage was enhanced due to this...
Fig. 4.13. Effect of exogenous ethyleneglycol tetraacetic acid (EGTA) and proline (L-Pro) applied alone and simultaneously on total chlorophyll contents (A) and Chl a/b ratio (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. *n* = 9 ± s.d.

Initial (0 d) values:
Total chlorophyll: 3.36 ± 0.04 mg g⁻¹ FW
Chl a/b ratio: 3.08 ± 0.11

* Fragile and very thin leaves
Fig. 4.14. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous ethyleneglycol tetraacetic acid (EGTA) and proline (L-Pro), applied individually or in combination. Leaves treated with EGTA and/or L-Pro for 5 d in darkness.
Fig. 4.15. Effect of exogenous ethyleneglycol tetraacetic acid (EGTA) and proline (L-Pro) applied alone and simultaneously on free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. *n* = 9 ± s.d.

Initial (0 d) values:
Free proline: 0.24 ± 0.06 μmol g⁻¹ FW
MDA: 0.088 ± 0.005 μmol g⁻¹ FW
K⁺ leakage: Not detected
* Not detected
treatment. Proline induced promotion of K⁺ leakage was strongly enhanced in the presence of EGTA (Fig. 4.15C).

Interaction between proline and Ca²⁺ channel blockers (Nifedipine, Verapamil)

Nifedipine effects on chlorophyll levels of senescing excised *T. majus* leaves differed from those of EGTA and EDTA in that with nifedipine senescing leaves retained more chlorophyll than the control. Further, proline-dependent enhanced loss of chlorophyll was reversed by a simultaneous application of nifedipine (Fig. 4.16 A, 4.17). The Chl a : Chl b ratio was not altered by lower nifedipine concentrations (0.001, 0.01 mM). The marginally lowered Chl a : Chl b ratio due to 10 mM proline was enhanced to the control levels by simultaneously applied nifedipine (Fig. 4.16 B).

Senescence dependent accumulation of proline in the leaves was not altered by the application of nifedipine. On the other hand, the enhanced leaf proline accumulation due to 10 mM proline was brought down by nifedipine in a concentration dependent manner. For example, leaves treated with proline (10 mM) + nifedipine (.01 mM) contained 50% less endogenous proline than the leaves treated with proline (10 mM) alone (Fig. 4.18A).

The MDA contents of leaves were enhanced by 0.001 mM and reduced by 0.01 and 0.1 mM nifedipine. MDA increase due to proline (10 mM) was lowered at 0.1 mM nifedipine. (Fig. 4.18B). The K⁺ leakage from leaves was not stimulated by nifedipine until 0.01 mM; a further increase in concentration to 0.1 mM strongly promoted the K⁺ loss. Further, the proline-
Fig. 4.16. Effect of exogenous nifedipine (Nif) and proline (L-Pro) applied alone and simultaneously on total chlorophyll contents (A) and Chl a/b ratio (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. n = 9 ± s.d.

Initial (0 d) values:
- Total chlorophyll: 2.97 ± 0.1 mg g⁻¹ FW
- Chl a/b ratio: 2.93 ± 0.14
Fig. 4.17. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous nifedipine (Nif) and proline (L-Pro), applied individually or in combination. Leaves treated with Nif and/or L-Pro for 5 d in darkness.
Fig. 4.18: Effect of exogenous nifedipine (Nif) and proline (L-Pro) applied alone and simultaneously on free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. *n* = 9 ± s.d.

Initial (0 d) values:
Free proline: 0.25 ± 0.03 μmol g⁻¹ FW
MDA: 0.086 ± 0.002 μmol g⁻¹ FW
K⁺ leakage: Not detected
induced stimulation of K\(^+\) leakage was intensified by nifedipine (0.1 mM, Fig. 4.18C).

The effects of verapamil on leaf senescence in \(T. \text{majus}\) differed from those of nifedipine in that verapamil, when applied individually in a range of 0.001 to 0.1 mM, had no effect on leaf senescence (chlorophyll loss). However, verapamil reversed the acceleration of senescence caused by 10 mM proline in a concentration dependent manner. A simultaneous application of 0.1 mM verapamil could totally abolish the senescence acceleration due to 10 mM proline (Fig. 4.19A, 4.20). The Chl a : b ratio fluctuated within a narrow change in response to proline and verapamil, applied either individually or in combination. No specific pattern was evident (Fig. 4.19B).

Although verapamil applied individually did not influence the leaf senescence pattern (chlorophyll loss) of \(T. \text{majus}\), proline accumulation due to dark incubation of leaves, was reduced by verapamil particularly at the highest applied concentration (0.1 mM). The leaves at 0.1 mM verapamil contained 40% proline that of control. Exogenous proline induced enhancement of endogenous proline was also suppressed markedly by the simultaneous application of verapamil. For example, 10 mM proline +0.1 mM verapamil - treated leaves contained 22% proline as compared to the leaves treated with 10 mM proline only (Fig. 4.21A).

The MDA contents of senescing leaves were lowered by verapamil in a concentration dependent manner. Also, the exogenous proline-induced enhancement of MDA contents was reduced by verapamil in a concentration dependent manner. For example, MDA contents of leaves
Fig. 4.19. Effect of exogenous verapamil (Ver) and proline (L-Pro) applied, alone and simultaneously on total chlorophyll contents (A) and Chl a/b ratio (B) of senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. $n = 9 \pm$ s.d.

Initial (0 d) values:
Total chlorophyll: $3.15 \pm 0.09$ mg g$^{-1}$ FW
Chl a/b ratio: $2.7 \pm 0.11$
Fig. 4.20. Senescence of excised *Tropaeolum majus* leaves as affected by exogenous verapamil (Ver) and proline (L-Pro), applied individually or in combination. Leaves treated with Ver and/or L-Pro for 5 d in darkness.
Fig. 4.21. Effect of exogenous verapamil (Ver) and proline (L-Pro) applied alone and simultaneously on free proline contents (A), malondialdehyde (MDA) contents (B) and K⁺ leakage (C) from senescing excised leaves of *Tropaeolum majus* in darkness.

Data after 5 d of dark incubation. n = 9 ± s.d.

Initial (0 d) values:
Free proline: 0.19 ± 0.02 μmol g⁻¹ FW
MDA: 0.075 ± 0.001 μmol g⁻¹ FW
K⁺ leakage: Not detected
* Not detected
treated with 10 mM proline + 0.1 mM verapamil were 40% of those in 10 mM proline (alone) – treated leaves (Fig. 4.21B). K⁺ leakage was not always connected with the degree of leaf lipid peroxidation. For example, despite the reduced MDA contents, K⁺ leakage actually increased with verapamil treatment particularly at 0.01 mM concentration. The proline induced stimulation of K⁺ leakage was further enhanced by 0.01 mM verapamil (Fig. 4.21C).