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# LEAF AND NODULE SENESCENCE IN CHICKPEA (*CICER ARIETINUM* L.) AND THE ROLE OF PLANT GROWTH REGULATORS

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**Abstract**

The present investigation was aimed to study the effect of Kinetin and Abscisic acid (ABA) on leaf and nodule senescence in chickpea (*Cicer arietinum* (L.)) cv. CM88, in relation to the changes in the endogenous level of abscisic acid (ABA) and indole-3-acetic acid (IAA). Studies was carried out for study the 1 involvement of hormonal and non-hormonal signal factors involved in nodule senescence, 2 The critical period in the development stages for the initiation of nodule senescence. 3 The correlation of leaf senescence with nodule senescence. The effects of the hormones were studied at four developmental stages; vegetative stage, flowering stage, early pod filling stage and late pod filling stage. The seeds of chickpea (*Cicer arietinum* (L.) cv. CM88 were soaked for 6h prior to sowing in aqueous solution of Kinetin and Abscisic acid each at $10^{-5}$ M. Both the hormones (at $10^{-6}$ M) were also applied as foliar spray during the vegetative phase of pant (4 weeks after sowing). The plants were allowed to grow under natural conditions. Increase in protein and sugar content of plant leaves was related with the increase in nodule activity at the flowering and early pod filling stages, thereafter, degradation of chlorophyll and protein content became more pronounced in old leaves as compared to young leaves. Kinetin at $10^{-5}$M was more effective than kinetin at $10^{-6}$M to delay leaf and nodule senescence. ABA ($10^{-5}$M) was effective in enhancing leaf and nodule senescence resulting in significant decrease in plant growth and yield as compared to the control. The effect of ABA in enhancement of leaf and nodule senescence is mediated possibly *via* the increased proline content of leaves and decrease in IAA and kinetin level. The exogenous application of kinetin decreased the endogenous level of ABA in the treated plants but elevated the IAA content of the root significantly at the early pod filling stage. Nodule senescence coincided with leaf senescence and appears to be correlated with the degradation of chlorophyll and protein content as well as the sugar assimilation in the host plant which in turn delay the degeneration of pink bacteroids tissue of nodules and the nitrogenase activity.

## Introduction

The close relationship between legume, *Rhizobium* and the environment governs $N_2$-fixation by the symbiotic association. There are many regulatory factors that operate directly or indirectly to influence nodule maturation. (Vinecent *et al*., 1980) have suggested a possible role for plant growth regulators formed by either partner to symbiosis in promoting or retarding nodule senescence. The plant hormones auxin and cytokinin have been shown to participate in the fundamental responses of nodule morphogenesis. Senescence has been reported to coincide with the pod-filling stage of reproductive growth (Anil *et al.*, 1985).

In soybean, cytokinins coming from the root via xylem are known to delay leaf senescence and their decline may be important in the senescence of soybean plants during pod development (Nooden *et al.*, 1990). Evidence suggest that it may not be the concentration of single hormone that is important in delaying or initiating the senescence process, but rather, the concentration of hormones relative to each other (Joyce & Thomas 1980).

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Studies were carried out to determine the effect of applied growth regulators, Kinetin and Abscisic acid on leaf and nodule senescence. Changes in endogenous ABA level and some biochemical contents associated with senescence were also determined.

## Materials and Methods

**Plant material and growing condition:** Seeds of chickpea (*Cicer arietinum* (L)) cv. CM88 were obtained from National Agricultural Research Center (NARC), Islamabad. The seeds were separately soaked with kinetin and Abscisic acid at $10^{-5}$M for 6h. Both hormones (at $10^{-6}$ M) were applied as foliar spray during vegetative phase) 4 weeks after sowing. Spray was made using plastic sprayer during 1000h-1200h.

The seeds were moistened in 48% sucrose solution prior to inoculation with *Rhizobium leguminosarum* (strain TAL-11480) and mixed thoroughly to uniform coating prior to sowing. Inoculants density used was $10^6$ cells per ml seed. Thereafter, seeds were sown in earthen pots (measuring 25x18cm$^2$) containing soil and organic matter (2:1) and allowed to grow under natural conditions. The plants were supplied with half strength Hoagland's nutrient solution once a week.

All the measurement were made at four growth stages, measured in weeks after sowing (WAS); vegetative (8WAS), flowering (16 WAS), early pod filling (18WAS) and late pod filling stages (20WAS) except ABA which was determined at vegetative and late pod filling stage.

The chlorophyll content of young and old leaves of plants at different stages were determined by the method of Arnon (1949) as modified by Kirk (1968). Absorbance of the extract was read at 645 nm (chlorophyll a) and at 663 nm (chlorophyll b) against 80% (v/v) acetone blank.

Protein content of young leaves (5-7 leaves from top) and old leaves (lower fully mature leaves) were assayed according to the methods of Peterson (1977), which is a modification of Lowry *et al*., (1951). A standard curve was plotted using known concentrations of BSA.

Sugar estimation of young and old leaves (fresh) was made following the method of Dubo *et al*., (1956) as modified by Johson *et al*., (1966). The absorbance of each sample was recorded at 420 nm and was calculated with reference to standard curve of glucose. Proline content of fresh young and old leaves was determined according to the method of Bates *et al*., (1973). Diameter of pink bacteroid tissue of thin section (5ul) of nodules was examined using light microscope at 4x magnification (Gretchen 1967).

Acetylene reduction assay of nodules, a test for nitrogen fixation was conducted following the method of Larue & Kurz (1973). The absorbance for ethylene ($C_2H_2$) was measured at 412 nm. A calibration curve was prepared using different volumes of ethylene gas.

Extraction and purification for endogenous ABA from fully expanded leaves of the control and treated plants at vegetative stage (8WAS) and at late pod filling stage (20WAS) was made according to the method of Kettner & Döerffing (1995). The leaves were freeze-dried, tritiated ABA (DL-cis trans - [$H^3$] ABA), specific activity 237 TBq/mmol$^{-1}$ (Radio chemical Center, Amersham International Buckinghamshire UK) was added as internal standard at 100 cpm to each sample during homogenization to account for the purification efficiency.

ABA analysis was carried out by HPLC (Model UNICAM 200, England) equipped with Particil-5 (P5-4659) column, and UV detector set at 254 nm. Acetonitrile and methanol (70:30) was used as solvent system, at a flow rate of 0.8 ml/minute. The purification efficiency varied between 60-70%.

**Results and Discussion**

The results showed that the young leaves showed higher chlorophyll content as compared to the old leaves (Table 1). A significant decrease in chlorophyll content was noted at the flowering and early pod filling stages in both the young and the old leaves. The decrease in chlorophyll content started much early (16 WAS) in old leaves as compared to 20 weeks after sowing in case of young leaves. The magnitude of decrease in chlorophyll content due to ABA was more in the old leaves as compared to that in young leaves possibly due to high endogenous ABA The stages of plants growth, treatments and leaf age, interacted significantly ($p<0.001$) (Table 1).

Kinetin at $10^{-5}$ M caused higher increase in chlorophyll content in the young and the old leaves at all stages of plant growth as compared to $10^{-6}$ M. The effect of kinetin was more in old leaves at early pod filling stage, at all other stages young leaves responded more to kinetin application. This indicates that perhaps kinetin helps to combat the effect of endogenous increase in ABA during this period. Early pod filling stage appears to be the critical period when senescence starts.

ABA at $10^{-5}$ M significantly decreased the chlorophyll content as compared to the control. The maximum decrease being noted with ABA ($10^{-5}$ M) treatment at the early pod filling stage and the effect was more pronounced in the old leaves as compared to the young leaves.

Wittenbach *et al.,* (1980); Grover *et al.,* (1985) and Congming & Jianhuazhang (1998) have reported that senescence of leaves was initiated after flowering and progressed. The senescence signal was transported basipetaly and accordingly the span of the leaf layer in the canopy tended to be longer toward the top of the plant at harvest (Sanetra *et al*., 1998).

ChaloupKova & Smart (1994) reported that the application of ABA significantly decreased chlorophyll content. ABA application enhances senescence more in old leaves as reported by (Samet & Sinclair 1980; Imre *et al.,* 1981) whereas young leaves were less affected by ABA. The ABA was reported less synthesized in young tissue as compared to mature tissues (Zeevart & Boyer, 1984).

Hajouj *et al*., (2000) reported that cytokinin delay the initiation of leaf senescence and bring about the promotion of photosynthetic activity mainly by increasing the chlorophyll content (Marek & Stewart 1992).

The protein content of leaf tissues was increased during the plant growth, being highest at the flowering stage (16WAS) in the young as well as in the old leaves (Table 2). Subsequent to the flowering stage, a significant decrease in protein content was noted in the young as well as the old leaves, the decrease being significantly higher in young leaves, thereafter at late pod filling stage protein content was increased both in young and old leaves, the increase was greater in the young leaves possibly due to the degradation or hydrolysis of conjugated protein.

The stages of plant growth and leaf age had significant effect on protein content in plant leaves; their interaction with different treatments was highly significant at $p<0.001$ (Table 2).

At late pod filling stage, kinetin increased the protein content of both the young and the old leaves as compared to the control. ABA at $10^{-5}$ M significantly decreased the protein content over control. But at the late pod filling stage the protein content was higher, possibly coming from the hydrolysis of conjugated proteins, the effect being more pronounced in young leaves. The increase in protein content following Kinetin induced increase in protein content was less in late pod filling stage, possibly the kinetin has counteracted the ABA induced hydrolysis of conjugated proteins. The significant decrease of protein content noted at late pod-filling stages in old leaves was concomitant with increase of protein content in young leaves this is possibly due to transfer of stored protein from old leaves to young leaves. Wollaston (1997) suggested that protein content in leaves can be rapidly degraded according to the need by other plant tissues.

Kinetin may recover the decline of protein during senescence (Mancera *et al*., 1999). It is proposed that kinetin might have been implicated directly in the process of regulating protein transcription and translation (Brinegar 1994). ABA decreased the protein content in young and old leaves at all growth stages, the decrease was more in old leaves at late pod filling stage possibly by ABA induced protease production in plant leaves (Rock & Quatrano, 1996). The ABA perhaps affected protein content by decreasing its synthesis and/or enhancing its degradation (Rock & Quatrano 1996).

Proline content was increased and remained high till early pod filling stage (18WAS) after which it decreased significantly (Table 3). The magnitude of increase was higher in old leaves as compared to the young leaves at all growth stages except the late pod filling stage. The reduction in proline content at the late pod filling stage was greater in the old leaves as compared to the young leaves, perhaps due to greater osmotic adjustment needed for its growth. The interaction of growth stages and leaf age with different treatments was highly significant ($p<0.001$) Table 3

The application of kinetin reduced the phenological increase in proline content in the young and the old leaves at all growth stages. The effect of kinetin in decreasing the proline content was higher at the early pod filling stage (18WAS) in the young leaves as compared to the old leaves.

ABA increased the proline content over the control in the young and the old leaves at all growth stages. The effect of ABA was higher in old leaves as compared to young leaves. ABA is involved in the accumulation of proline as osmoregulant (Pospisilova 2003; Hose *et al*., 2000).

At late pod filling stage, the maximum sugar content was reported in both the old and the young leaves (Table 4). The old leaves exhibited much higher sugar content than that of young leaves. Treatments and stages of plant growth interacted highly significantly ($p<0.001$).

Kinetin treatment significantly increased the sugar content of leaves at all stages more so at late pod filling stage (20WS). The effect of kinetin was greater in old leaves as compared to young leaves. The decrease in sugar content in kinetin treatments at flowering stage was greater in the old leaves as compared to the young leaves.

The application of ABA reduced the sugar content in the plant leaves non-significantly as compared to the control at all growth stages. At the late pod filling stage, ABA ($10^{-5}$ M) significantly decreased the sugar content of the young and the old leaves, though the value was higher as compared to that of previous stage. Sugar is possibly transported from mature leaves to young developing leaves (Brandner *et al*., 1984). Erwin (1996) reported that cytokinin balance of plants resulted in disproportionate distribution of assimilates in favor of the cytokinin-enriched shoot. Application of ABA reduced sugar content in young and old leaves at all growth stages but the effect was more evident at flowering stage. The decrease was more in old leaves perhaps due to

increased ABA synthesis in old leaves (Samet & Sinclair 1980). Sugars are also considered to play important role in osmotic adjustment, which is widely regarded as an adaptive response to stress condition (Kameli & Loesel, 1995).

A significant increase of ABA content in the control treatment (Table 7) was noted at the late pod filling stage (20 WAS) as compared to the vegetative stage (8WAS). The interaction between treatments and plant growth stages was highly significant in affecting the endogenous level of ABA.

Kinetin at $10^{-5}$ M concentration significantly decreased the endogenous level of ABA as compared to the control at vegetative phase but no significant difference from control was observed at late pod filling stage. Low concentration of kinetin ($10^{-6}$ M) was ineffective at vegetative phase rather stimulated ABA production over control at late pod filling stage. The exogenous application of ABA induced accumulation of endogenous ABA at both phases; the extent of increase was greater with higher concentration of ABA.

The effect of treatments and growth stages and their interaction were highly significant ($p<0.001$) Table 7. The significant increase in endogenous level of ABA in old leaves at late pod filling stage can be attributed possibly to the release from the conjugated form accompanied by increased synthesis; old leaves are more active in synthesizing ABA than young leaves (Lindoo & Nooden 1978; Neales *et al.*, 1989). The decrease in the endogenous level of ABA due to kinetin treatment has previously been reported (Chin & Beevers, 1970). Pospisilova *et al*., (2005) demonstrated that ck might suppress xanthin dehydrogenase activity or enhance conversion of ABA to phaseic acid. The antagonism between Cks and ABA may be the result of metabolic interactions. Cks share at least in part, a common biosynthetic origin with ABA (Cowan *et al.*, 1999).

Both diameter of pink bacteroid tissue and nitrogenase activity increased during the development stages in all the treatments to reach its maximum rate at the early pod filling stage followed by sharp decrease at late pod filing stage. Kinetin significantly increased the diameter of the pink bacteroids tissue and nitrogenase activity as compared to the control at different stages of plant growth (Tables 5 & 6). Kinetin at $10^{-5}$M was more effective in increasing the diameter of bacteroid tissue and subsequently the nitrogenase activity.

ABA treatments showed decrease in the bacteroid tissue and nitrogenase activity at all stages being maximum at late pod filling stage. The observed decrease in nodule activity at early pod filling stage was reported by Moro *et al*., (1992). The decrease in nitrogenase coincided with leaf senescence (De Lima *et al*., 1994). The decline in nitrogen fixation rate at late pod filling stage is possibly due to carbohydrate deprivation of nodule as photo-assimilate are partitioned to pods at the expense of nodule, see Vessey *et al*., (1988).

Exogenous application of kinetin enhances nitrogenase activity of nodules at all growth stages, (Dayal & Bharti, 1991; Garg *et al*., 1995). Bano & Hillhman (1986) have reported that ABA treatment resulted in reduction in the volume of functional bacteroid tissue per nodule perhaps due to formation of lignin ring. This ABA induced senescence of pink bacteroid tissue may be assigned to the deprivation of photosynthate to the nodules as both chlorophyll content and sugar accumulation was decreased as early signal of leaf senescence.

The results (Table 8) indicated a significant decrease in lAA content at the flowering stage (16 WAS) followed by a sharp increase at the early pod filling stage (18 WAS) and a significant decline at the late pod filling stage (20 W AS). The results indicated the significant effect of treatments and growth stages on the lAA content.

**Table 5. Effect of plant growth regulators on diameter of pink bacteroid tissue ($mm^3$ $plant^{-1}$ $h^{-1}$) in root nodule of *Cicer arietinum* L. cv. CM 88 at different plant growth stages.**

| Treatments | Weeks after sowing | | | | Mean |
|---|---|---|---|---|---|
| | 8 | 16 | 18 | 20 | |
| Control | 1.05 jk | 1.77 c-e | 1.88 b-d | 1.25 hi | 1.49 C |
| Kinetin ($10^{-5}$ M) | 1.25 hi | 2.04 ab | 2.17 a | 1.60 ef | 1.76 A |
| Kinetin ($10^{-6}$ M) | 1.11 ij | 1.92 bc | 2.00 ab | 1.38 gh | 1.60 B |
| ABA ($10^{-5}$ M) | 0.93 j-l | 1.70 d-f | 1.65 ef | 0.95 j-l | 1.31 D |
| ABA ($10^{-6}$ M) | 0.85 l | 1.57 f | 1.52 fg | 0.89 kl | 1.21 E |
| Mean | 1.04 C | 1.80 A | 1.84 A | 1.21 B | |

All such means which share a common English letter are statistically similar other wise are different at $\alpha = 0.05$.

**Table 6. Effect of plant growth regulators on nitrogenase activity (nmol $C_2H_4$ $plant^{-1}$ $h^{-1}$) in root nodule of *Cicer arietinum* L. cv. CM88 at different plant growth stages.**

| Treatments | Weeks after sowing | | | | Mean |
|---|---|---|---|---|---|
| | 8 | 16 | 18 | 20 | |
| Control | 2.35 jk | 5.39 e-h | 8.53 c | 2.4 d-g | 4.7 C |
| Kinetin ($10^{-5}$ M) | 4.31 g-i | 8.63 c | 17.70 a | 3.2 d-f | 8.5 A |
| Kinetin ($10^{-6}$ M) | 3.32 ij | 6.63 de | 13.6 b | 2.7 d-g | 6.5 B |
| ABA ($10^{-5}$ M) | 1.86 jk | 4.06 hi | 7.08 d | 1.7 f-i | 3.7 D |
| ABA ($10^{-6}$ M) | 1.57 k | 3.14 i-k | 6.28 d-f | 1.6 f-i | 3.19 D |
| Mean | 2.68 C | 5.57 B | 10.6 A | 2.3 B | |

All such means which share a common English letter are statistically similar other wise are different at $\alpha = 0.05$.

**Table 7. Endogenous ABA in leaf samples (µg/g freeze dried leaf) in *Cicer arietinum* L. cv. CM 88 at two stages of plant growth.**

| Treatments | Weeks after sowing | | Mean |
|---|---|---|---|
| | 8 | 20 | |
| Control | 184.0 g | 473.4 cd | 328.8 D |
| Kinetin ($10^{-5}$ M) | 101.4 h | 450.4 d | 276.0 E |
| Kinetin ($10^{-6}$ M) | 178.6 g | 708.8 b | 443.8 B |
| ABA ($10^{-5}$ M) | 348.6 e | 858.2 a | 603.4 A |
| ABA ($10^{-6}$ M) | 254.4 f | 485.4 c | 369.8 C |
| Mean | 213.4 B | 595.2 A | |

All such means which share a common English letter are statistically similar other wise are different at $\alpha = 0.05$.

**Table 8. DMRT of means of IAA content (mg g□1 root FW) of Cicer *arietinum* L. cv. CM88 at different plant growth stages and the effect of plant growth regulators.**

| Treatments | ---------- Weeks after sowing ---------- | | | | Mean |
|---|---|---|---|---|---|
| | 8 | 16 | 18 | 20 | |
| Control | 3.77 c | 0.7 c | 62.28 b | 1.14 c | 16.99 A |
| Kinetin ($10^{-5}$ M) | 5.1 c | 0.79 c | 91.1 a | 1.3 c | 24.62 A |
| Kinetin ($10^{-6}$ M) | 4.82 c | 0.79 c | 64.65 b | 1.14 c | 17.874 A |
| ABA ($10^{-5}$ M) | 1.93 c | 0.06 c | 6.66 c | 1.12 c | 2.54 B |
| ABA ($10^{-6}$ M) | 1.93 c | 0.7 c | 6.75 c | 1.12 c | 2.72 B |
| Mean | 3.59 B | 0.7 B | 46.25 A | 1.14 B | |

All such means which share a common English letter are statistically similar other wise are different at $\alpha = 0.05$.

**Table 9. Effect of plant growth regulators on the yield of *Cicer arietinum* L. cv. CM 88 harvested at edible pod stage.**

| Treatments | Grain weight (g) | Weight of 100 grains (g) | No. of pods plant $^{-1}$ |
|---|---|---|---|
| Control | 3.1 B | 77.2 B | 3.0 BC |
| Kinetin ($10^{-5}$ M) | 5.2 A | 88.9 A | 5.3 A |
| Kinetin ($10^{-6}$ M) | 4.3 B | 80.2B | 4.3 B |
| ABA ($10^{-5}$ M) | 2.3 D | 61.8 C | 2.6 E |
| ABA ($10^{-6}$ M) | 3.5 C | 64.2 C | 3.5 D |

All such means which share a common English letter are statistically similar otherwise are different at ¦Á = 0.05.

Kinetin at $10^{-5}$M had significantly higher lAA content than control. ABA decreased the accumulation of lAA during different stages as compared to the control. The decrease was non significant at the early pod filing stage, however ABA treatments significantly decreased the lAA content at late pod filling stage as compared to the control. The ABA had the potential to antagonize other growth hormones like IAA (Greg *et al*., 1991) and cytokinins (Chaloupkova & Smart, 1994). Kinetin application significantly increased the yield as compared to the control. The Kinetin at $10^{-5}$M being more effective (Table 9). In contrast the yield decreased when ABA was applied. The magnitude of inhibition was more when ABA was applied at $10^{-5}$M as seed soaking than that of foliar spray at $10^{-6}$M. Kinetin-enhanced yield in chickpea was reported by Atkins & Pigeaire (1993), which was attributed to kinetin-induced increase in chlorophyll and protein content. ABA has been found to be associated with decrease in grain yield of maize (Sanguineti *et al*., 1999).

## Conclusion

Chlorophyll degradation is the first symptom which starts to show up at early pod filling stage followed by protein degradation. Higher concentration of kinetin (at $10^{-5}$M) is needed to combat the endogenous increase of ABA and delay senescence. The mechanism of kinetin induced inhibition in leaf and nodule senescence appears to be mediated by increase in leaf sugar content particularly at late pod filling stage and more in old leaves, which is the main donor of photosynthate to nodules. Consequently, the degeneration of pink becteroid tissue the active site of nitrogen fixation was delayed resulting in higher nitrogenase activity. During leaf senescence, study should be extended

to monitor the partitioning of assimilates from leaves to the developing pods and seeds as well as from the leaves to nodules.

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