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Changes in the inorganic status and enzyme activities in senescent leaves of chickpea, *Cicer arietinum* L.

CHANDRASHEKHAR V. MURUMKAR, PRAKASH D. CHAVAN

Botany Department, Shivaji University, Kolhapur 416 0004, India
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Abstract

The changes in the level of some inorganic constituents and the activities of some important enzyme systems in senescent leaves of chickpea (*Cicer arietinum* L.) have been studied. In senescent leaves, a marked decline in the potassium and phosphorus contents was evident which was accompanied by the accumulation of calcium, silicon, chloride and manganese. Leaf senescence was accompanied by a great increase in hydrolytic processes, as revealed by the increase in the activities of acid phosphatase, alkaline phosphatase, ATPase, inorganic pyrophosphatase and 3-phosphoglycerate phosphatase. The activities of nitrogen metabolism enzymes, namely nitrate reductase, nitrite reductase, glutamine synthetase and alanine aminotransferase, and of photorespiratory enzymes — phosphoglycolate phosphatase, glycolate oxidase and catalase, were lower in senescent leaves. Leaf senescence was further associated with an increase in the activities of peroxidase and polyphenol oxidase, a considerable depression in pyruvate kinase activity, and a slight elevation in aldolase activity.

Key words: chickpea, Cicer arietinum, senescence, inorganic constituents, nitrogen metabolism, photorespiration

INTRODUCTION

It is reported by some workers that as much as a 3% increase in yield per day is possible by regulation of leaf senescence in crops like wheat (Hardy et al. 1978). However, in searching for possible methods of regulation of leaf senescence, it is essential to characterize various biochemical events during the process. Chickpea (*Cicer arietinum* L.) is one of the major legume crops of semi-arid tropic. According to Sheldrake (1979), in field, chickpea leaf senescence is affected by three main factors: water stress, heat stress and internal physiological factors. Internal physiological

factors have not been clearly identified. The present work comprises an attempt to characterize and assess some physiological changes during leaf senescence in this crop.

MATERIAL AND METHODS

Like most legumes, chickpea leaf senescence is clearly reveled by distinct yellowing. Senescent (4 weeks old, yellow) as well as fully expanded mature leaves (2 weeks old, green) were collected at 12.00 noon during February (a major chickpea cultivation period in India) from the same plants raised in chickpea fields. The leaves were brought to the laboratory in air-tight chambers, washed to remove surface dust and blotted to dry.

Analysis of **inorganic constituents** from the acid digest of oven-dried leaf samples was performed according to methods described elsewhere (Chavan and Karadge 1980).

Enzyme assay. The cell-free preparations and assay of various enzymes from the leaves collected from the field were made using the methods given below. Each enzymatic assay was in triplicate.

Phosphatases. Enzyme acid phosphatase (EC 3.1.3.2) was extracted using 100 mM acetate buffer (pH 5.0) and the extract was centrifuged at $10000 \times g$ to yield a supernatant (enzyme) (Melachalan 1980). 4 cm^3 assay mixture contained 100 mM acetate buffer pH 5.0, p-nitrophenol phosphatase (0.1 $mg \cdot cm^3$), enzyme and 1.68 N NaOH (killing agent). The p-nitrophenol color complex formed was measured colorimetrically at 400 nm wave length. For alkaline phosphatase (EC 3.1.3.1) (Weimberg 1970) and inorganic pyrophosphatase (EC 3.6.1.1) (Rauser 1971), the enzyme was extracted with 100 mM Tries-HCl buffer (pH 8.0) containing 1000 mM KCl, 10 mM EDTA and 0.4% β-mercaptoethanol and centrifuged at $10000 \times g$ to yield a supernatant (enzyme). Alkaline phosphatase activity was determined in an assay mixture containing 1 cm^3 of 100 mM Tris-HCl (pH 7.5), 0.1 cm^3 of 50 mM $MgCl_2$, 1.7 cm^3 H_2O, 0.2 cm^3 extract and 0.1 cm^3 of 200 mM p-nitrophenol phosphate. The colour complex was estimated colorimetrically at 400 nm. Inorganic pyrophosphatase was assayed from one cm^3 assay mixture containing 100 mM Tris-K phthalate (pH 8.7), 20 μmol $Na_2P_2O_7$, 40 μmol $MgCl_2$, 10% TCA (killing agent) and 0.5 cm^3 enzyme source. Liberated P_i was estimated according to Fiske and Subbarow (1925).

Enzymes of nitrogen metabolism. For *in vivo* measurement of nitrate reductase (EC 1.6.6.2) and nitrate reductase (EC 1.7.7.1) the method of Evans (1982) and Ramarao et al. (1983) was followed with slight

modifications. Freshly cut leaf discs (0.5 g) were suspended in 5 cm^3 of 100 mM phosphate buffer (pH 7.5) containing 20 mM KNO_3, 5% propanol and two drops of chloramphenicol (0.5 $mg \cdot cm^3$) in sealed jars in darkness (for nitrite reductase KNO_3 was replaced by KNO_2 and assay was conducted in light). Liberated NO_2^- (unconsumed in case of nitrite reductase) was estimated by reaction with 1% sulfanilamide and 0.2% N-1-naphthylethylene-diamine hydrochloride (NEEDA) colorimetrically at 540 nm. Glutamate oxaloacetate transferase (GOT) (EC 2.6.1.1) and alanine amino transferase (AAT) (EC 2.6.1.2) were estimated according to Green et al. (1945). The enzyme was extracted with 100 mM Tris-HCl buffer followed by centrifugation at $10000 \times g$ to yield a supernatant which served as the enzyme source. The assay mixture contained 200 mM aspartate, 200 mM α-keto-glutarate, 10 mM DNPH, 0.75 N NaOH and 0.25 cm^3 enzyme. The optical density of the phenylhydrazone formed was measured at 504 nm. The assay mixture of AAT contained alanine instead of aspartate. Glutamine synthetase (EC 6.3.1.2) was extracted in 50 mM imidazole buffer (pH 7.8) containing 0.5 mM EDTA, 1 mM dithiothreitol, 2 mM $MnCl_2$ and 20% glycerol followed by centrifugation at $10000 \times g$. Assay mixture (1 cm^3) contained 12.5 mM glutamate (sodium salt), 5.0 mM ATP (sodium salt) 10.0 mM $MgCl_2$, 6.0 mM hydroxylamine hydrochloride, 2.0 mM EDTA, 100.0 mM imidazole acetate and 0.2 cm^3 enzyme extract. The glutamyl hydroxymate formed was measured colorimetrically at 540 nm using ferric chloride reagent.

Enzymes of photorespiration. Glycolate oxidase (EC 1.1.1.26) was extracted with 100 mM Tris-HCl (pH 8.0), followed by centrifugation at $10000 \times g$ to yield supernatant (enzyme source) and assayed with one cm^3 assay mixture containing 100 mM Tris-HCl (pH 8.3), 100 mM phenylhydrazine hydrochloride, 100 mM cysteine, 100 mM glycolate and 0.5 cm^3 enzyme source. Phenylhydrazone formation was recorded spectrophotometrically at 346 nm (Hess and Tolbert 1967). For catalase (EC 1.11.1.6) (Herbert 1955) activity, 1 cm^3 enzyme (obtained from extraction of leaf tissue with 100 mM Tris-HCl, pH 8.0) was incubated with 450 mM H_2O_2. Liberated O_2 oxidized 10% KI and liberated I_2 was titrated against 0.01 N $Na_2S_2O_3$ using starch indicator. 3-Phosphoglycerate phosphatase (3 PGAP) (EC 2.7.2.3) and phosphoglycolate phosphatase (PGP) (EC 3.1.3.18) enzymes were extracted with 20 mM cacodylate buffer (pH 6.3) containing 1 mM EDTA followed by filtration and centrifugation at $10000 \times g$ to yield supernatant (enzyme source) (Randall et al. 1971). PGP was assayed using 10 μmol phosphoglycolate (pH 6.3). 1 mM $MgCl_2$, 1 cm^3 enzyme and 1 cm^3 10% TCA (for termination of reaction). PGAP was assayed in the same manner but at pH 6.9 without $MgCl_2$ and 3-phosphoglycerate was used as the substrate instead of phosphoglycolate. Liberated P_i in both the cases was measured by the method of Fiske and Subbarow (1925).

Respiratory enzymes. Pyruvate kinase (EC 2.7.1.40) was assayed from

an extract made using 100 mM Tris-HCl (pH 8.0) followed by centrifugation at 10000 × g to yield supernatant (enzyme) (Weidner and Salisbury 1974). One cm^3 assay mixture contained 15 mM PEP, 25 mM NADP, 500 mM KCl, 100 mM $MgSO_4$, 0.1 cm^3 enzyme, 0.0125% DNPH (2,4-dinitrophenyl hydrazine) and 0.6 N NaOH. The optical density of phenylhydrazone formed at 510 nm was taken as a measure of enzyme activity. The method of Akiva et al. (1971) was followed for aldolase (EC 4.1.2.13). The assay mixture contained 1 cm^3 50 mM Tris-HCl (pH 8.5), 0.2 cm^3 200 mM hydrazine sulphate, 0.1 cm^3 enzyme extract (in 100 mM Tris-HCl, pH 8.0) and 0.2 cm^3 20 mM fructose 1-6-diphosphate followed by 1 cm^3 10% TCA, 1 cm^3 0.125% DNPH and 5 cm^3 0.6 N NaOH. The phenylhydrazone derivative formed was recorded colorimetrically at 540 nm.

The method of Maehly (1954) was followed for determination of the enzyme peroxidase (EC 1.11.1.6), by extracting it with 100 mM phosphate buffer (pH 7.0), followed by centrifugation at 10000 × g to yield a supernatant (enzyme source). The reaction mixture contained 20 mM guiacol and 10 mM H_2O_2, 0.2 cm^3 enzyme; guiacol peroxidation was recorded colorimetrically at 470 nm.

For polyphenol oxidase (EC 1.10.3.1) (Sato and Hasegawa 1976), the enzyme was extracted with 100 mM phosphate buffer (pH 6.6) followed by centrifugation at 10000 × g to obtain a supernatant (enzyme source) and was assayed by following oxidation of 10 mM catechol in 100 mM phosphate buffer (pH 6.3) at 490 nm.

Proteins in all enzyme extracts were determined according to the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

There were marked alterations in the level of various inorganic constituents in senescent chickpea leaves (Table 1). The potassium and phosphorus contents were considerably lowered in senescent leaves and this can be attributed to their remobilization to the rapidly growing tissues (Hill 1980). An opposite trend is noticed in the case of calcium, as there was massive accumulation of it in senescent leaves. These observations recall the work of Waughman and Bellamy (1981) who observed accumulation of Ca in senescent leaves in several plant species. According to Ishizuka (1965), the mobility quotient of calcium is about zero, which is responsible for its deposition in old rice leaves. Magnesium, iron and manganese contents were a little higher in the senescent leaves than in mature leaves, while the sodium content was reduced in the senescent leaves. In senescent

leaves of chickpea, the silicon level was considerably higher than in the mature leaves (Table 1). This may also lead to a decrease in the potasium content as suggested by Soni et al. (1972) in the case of ageing of *Cyperus alternifolius* leaves. Chloride accumulation was also enhanced in senscent chickpea leaves (Table 1). These observations suggest that an overall

Table 1

Values of inorganic constituents (in Meq per 100 g dry tissue) of mature and senescent leaves of chickpea, *Cicer arietinum L.*

Inorganic constituent	Mature leaves	Senescent leaves
Potassium	85.037*	49.641
Calcium	181.736	579.788
Phosphorus	11.987	1.429
Magnesium	76.857	74.704
Manganese	1.907	2.948
Iron	1.219	2.353
Sodium	12.092	8.09
Chloride	7.106	12.352
Silica	11.05	29.228

* Each value is mean of three determinations.

ionic imbalance arises in senescent chickpea leaves, which in turn can affect various metabolic activities. These observations agree with the findings of Nimbalkar and Joshi (1974), Pathan and Nimbalkar (1979) and Gokhale et al. (1984) in sugarcane, *Alternanthera* and *Catharanthus roseus* leaves respectively.

The changes in enzyme levels during leaf senescence in chickpea leaves are recorded on Fig. 1. The absolute values of the specific activity of enzymes in mature leaves are depicted in Table 2.

The activities of enzymes involved in hydrolysis of phosphates (ATPase, acid phosphatase, alkaline phosphatase, 3-phosphoglycerate phosphatase and inorganic pyrophosphatase) were higher in senescent leaves (Fig. 1). The increase was particularly significant in the case of ATPase, acid phosphatase and inorganic pyrophosphatase. An increase in acid phosphatase involved in catabolic processes during senescence and ripening was found by De Leo and Sacher (1970) and Kar and Mishra (1976). The activity of acid inorganic pyrophosphatase was increased, but that of alkaline inorganic pyrophosphatase decreased during senescence in excised wheat leaves (Kao 1981) which is in contrast with our results. The increase in ATPase activity in senescent leaves can represent a decline in the ATP level.

In detached leaf segments an initial increase in the ATP level at the beginning of senescence was observed by Malik and Thimann (1980), but later the ATP level sharply declined.

In contrast to the above enzymes, the activities of enzymes such as nitrate reductase, nitrite reductase and glutamine synthetase were lower in senescent leaves (Fig. 1). This certainly affects the protein synthesizing

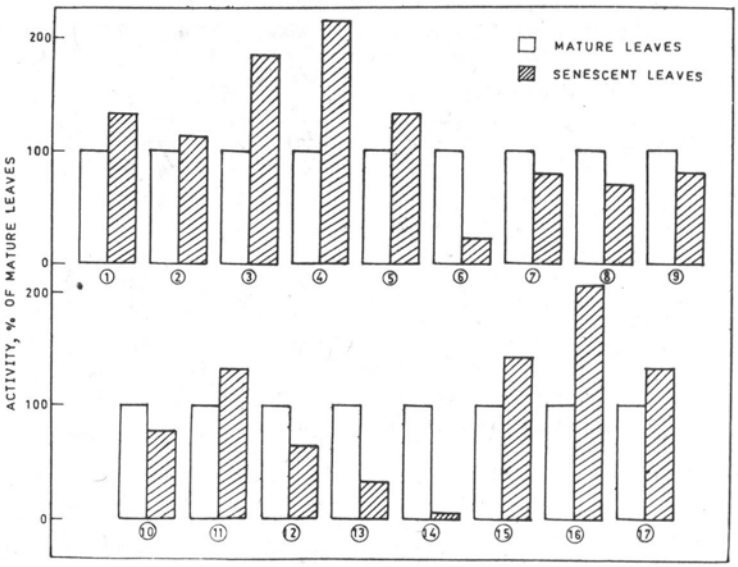

Fig. 1. Enzymatic changes in senescent chickpea (*Cicer arietinum*) leaves. 1 — acid phosphatase, 2 — alkaline phosphatase, 3 — inorganic pyrophosphatase, 4 — ATPase, 5 — 3-phosphoglycerate phosphatase, 6 — phosphoglycolate phosphatase, 7 — nitrate reductase, 8 — nitrite reductase, 9 — glutamine synthetase, 10 — alanine aminotransferase, 11 — glutamate oxaloacetate transferase, 12 — glycolate oxidase, 13 — catalase, 14 — pyruvate kinase, 15 — aldolase, 16 — peroxidase, 17 — polyphenol oxidase. Each value is mean of three determinations

machinery of the senescent leaves. A decrease in nitrate reductase during senescence has been reported by Teare et al. (1974), Khol et al. (1975) and Schlesier (1977). Storey and Beevers (1978) observed a decline in glutamine synthetase in pea during senescence. The alanine amino transferase activity was decreased during chickpea leaf senescence, while the opposite was true in the case of glutamate oxaloacetate transaminase (Fig. 1). A similar imbalance in the activities of transaminases was also found by Howard (1975); this can lead to a disturbance of the balance of keto acids and amino acids in the senescent leaves.

The activities of photorespiratory enzymes such as phosphoglycolate phosphatase, glycolate oxidase and catalase were strongly reduced in senescent

Table 2

Specific activity of enzymes in mature leaves of hickpea, *Cicer arietinum L.*

Enzyme	Enzyme activity
Acid phosphatase	1.913* △ O.D. h^{-1} mg^{-1} protein
Alkaline phosphatase	3.64 „ „
Inorganic pyrophosphatase	99.032 µg P_i liberated h^{-1} mg^{-1} protein
ATPase	33.37 „ „
3-Phosphoglycerate phosphatase	34.741 „ „
Phosphoglycolate phosphatase	111.411 „ „
Nitrate reductase	2.197 nmol NO_2 formed h^{-1} mg^{-1} protein
Nitrite reductase	5.166 nmol NO_2 broken down h^{-1} mg^{-1} protein
Glutamine synthetase	0.063 nmol glutamyl hydroxymate formed h^{-1} mg^{-1} protein
Alanine amino transferase	0.031 △ O.D. h^{-1} mg^{-1} protein
Glutamate oxaloacetate transferase	0.052 „ „
Glycolate oxidase	0.427 „ „
Catalase	0.695 mg H_2O_2 liberated min^{-1} mg^{-1} protein
Pyruvate kinase	6.656 △ O.D. h^{-1} mg^{-1} protein
Aldolase	4.7 „ „
Peroxidase	4.934 △ O.D. min^{-1} mg^{-1} protein
Polyphenol oxidase	0.166 △ O.D. h^{-1} mg^{-1} protein

* Each value is mean of three determinations.

chickpea leaves (Fig. 1). Patric et al. (1972) and Dezsi (1975) observed a decrease in glycolate oxidase activity with age in barley leaves. A decline with senescence in RuBP carboxylase/oxygenase activity, the key enzyme protein of photosynthesis and photorespiration, was observed by Callow (1974), Wittenbach (1978) and Secor et al. (1983). Catalase is generally involved in regulating the cell H_2O_2 level, which otherwise would prove toxic to the cell. A considerable decline in catalase activity (Fig. 1) will undoubtedly raise the H_2O_2 level in the senescent leaves. Omran (1976) suggested that cellular H_2O_2 can inhibit plant growth and development through destruction of IAA.

An increase in peroxidase and polyphenol oxidase activities in senescent chickpea leaves (Fig. 1) was observed, which agrees with the findings of Patra and Mishra (1979) in the leaves of eight monocot and eight dicot species. An increment of these two enzymes indicates a promotion of secondary metabolism during senescence. Peroxidase can bring about oxidation of auxin (Pilet et al. 1970) and hence its increase in senescent leaves may induce hormonal imbalance.

The activity of pyruvate kinase was considerably lowered in senescent chickpea leaves (Fig. 1). Leaf senescence is generally accompanied by considerable nutrient retranslocation (Hill 1980). An ionic regulation of pyruvate kinase has been established by Evans (1963). Hence, a decrease in pyruvate kinase can be attributed to possible shifts in ionic equilibria in senescent leaves. The significance of pyruvate kinase as a link between respiration and photosynthesis is discussed by Duggleby and Dennis (1973). The increase in aldolase activity in senescent leaves of chickpea (Fig. 1) also adds to such alterations. A similar enhancement of aldolase activity during ageing of potato discs was found by Sacher et al. (1972). It is evident from the foregoing account that leaf senescence in chickpea is accompanied by marked changes in enzymatic activities as well as in inorganic constituents and this can ultimately lead to modification of normal leaf metabolism at several levels.

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Zmiany zawartości składników nieorganicznych i aktywności enzymów w starzejących się liściach cieciorki, Cicer arietinum L.

Streszczenie

Badano zmiany poziomu niektórych składników nieorganicznych i aktywność enzymów niektórych ważnych szlaków metabolicznych w starzejących się liściach cieciorki (*Cicer arietinum* L.). Stwierdzono wyraźnie mniejszą zawartość potasu i fosforu, czemu towarzyszyło gromadzenie się wapnia, krzemu, chlorków i manganu. Starzenie się liści było związane z dużym nasileniem się procesów hydrolitycznych, co było widoczne w większej aktywności: kwaśnej fosfatazy, fosfatazy zasadowej, ATPazy, pirofosfatazy nieorganicznej i fosfatazy

3-fosfoglicerynianu. W starzejących się liściach stwierdzono mniejszą aktywność enzymów metabolizmu azotowego, takich jak: reduktaza azotanowa, reduktaza azotynowa, syntetaza glutaminowa, aminotransferaza alaninowa, ponadto — enzymów fotooddechowych: fosfatazy fosfoglikolowej, oksydazy glikolowej i katalazy. Starzenie się liści było również związane z większą aktywnością peroksydazy i oksydazy polifenolowej, z znacznym zmniejszeniem się aktywności kinazy pirogronianowej oraz z wyraźnym wzrostem aktywności aldolazy.