BIOLOGIA PLANTARUM 50 (4): 705-708, 2006

BRIEF COMMUNICATION

Gender-specific responses of *Piper betle* L. to low temperature stress: changes in chlorophyllase activity

N. KUMAR*, S. GUPTA and A.N. TRIPATHI

Betel Vine Biotechnology Laboratory, NBRI, Lucknow-226001, India

Abstract

Gender based differences in response to low temperature stress in leaf chlorophyll (Chl), and carotenoids (Car) contents and chlorophyllase (Chlase) activity were monitored in male (Kapoori Vellaikodi and Madras Pan Kapoori) and female (Bangla Mahoba, Desi Bangla and Kaker) betel vine landraces. Although female plants contained nearly two fold more Chl than male counterparts, the low temperature induced Chl loss was comparable, however, male plants showed higher Chl *a*/*b* ratio than females. Chlase activity increased due to cold stress in all the landraces. Male plants always showed higher activities of Chlase, which may be one of the reasons for the rather low Chl contents in male plants.

Additional key words: betel vine, chilling, chlorophyll, dioecy, shade plant.

In flowering plants the phenomenon of dioecy occurs in just 7 % of the known plant species distributed in many orders and families (Renner and Ricklefs 1995). Gender based differences of varying magnitudes in physiological properties and their responses to abiotic stresses have been shown in several plant species (Dawson and Bliss 1989, Marshall *et al.* 1993, Dawson and Geber 1999, Wang and Curtis 2001, Tyagi and Prakash 2004). Temperature is one of the most important abiotic factors controlling the global distribution of plant species. A common response to low- or high-temperature stress is the impaired biosynthesis (Van Hasselt and Strikwerda 1976, Feierabend 1977, Tewari and Tripathy 1999) and enhanced enzymatic degradation of chlorophyll (Chl). Chlorophyllase (Chlase; chlorophyll-chlorophyllido hydrolase, E.C. 3.1.1.14) is one of the oldest known plant enzymes reported to cause dephytylation of Chl (Stoll 1912). The enzyme has been studied extensively in a wide variety of plants including purple bacteria, blue green and green algae and leaves, fruits and seeds of higher plants (Tanaka *et al.* 1982, Shioi *et al.* 1991, Fernandez-Lopez *et al.* 1992, Nishiyama *et al.* 1994, Khalyfa *et al.* 1995). However, there are no reports on gender-based differences in Chlase and its response to abiotic stresses. We present our findings on gender-based differences in Chl and Chlase in *Piper betle* and its response to low temperature stress.

Piper betle landraces were collected from different regions of India and grown in the botanical garden of the National Botanical Research Institute, Lucknow. The plants were grown at 30 - 40 % of incident photosynthetically active radiation (PAR 400 - 600 µmol m^{-2} s^{-1}). Changes in chloroplast pigments and Chlase activity were monitored during October to March (2003 - 2004) covering the active, cold stress and active growth period. Fully expanded mature leaves were used and each data point represents an average of six measurements. Since the plants have explicit dioecy, three female landraces Bangla Mahoba (BM), Desi Bangla (DB) and Kaker and two male landraces, Kapoori Vellaikodi (KV) and Madras Pan Kapoori (MPK) were used.

Chloroplast pigments were extracted from single leaf disc (10 cm^2) in buffered cold 80 % acetone and the absorbance was read at 470.0, 646.8 and 663.2 nm (Lichtenthaler 1987) on GBC, Cintra 20 spectrophotometer. A leaf disc of 10 cm^2 was homogenized in cold extraction buffer containing 100 mM phosphate buffer (pH 7.0), 50 mM KCl, 5 mM sodium diethyl dithiocarbamate, 1 mM diethylenepentamine acetic acid, 0.24 % Triton X-100 and 15 % (m/m) pre-swollen

---

Received 23 September 2004, *accepted* 19 August 2005.

Abbreviations: Chl - chlorophyll, Chlase - chlorophyllase, Car - carotenoid, PAR - photosynthetically active radiation.

Acknowledgements: The work was supported by DST grant No. SP/SO/A42/95 DST, Govt. of India. Critical reading of the manuscript by Dr. Shanti S. Shrama, Bioscience HP Univ., Shimla is acknowledged. SG is recipient of CSIR research fellowship.

* Corresponding author; fax: (+91) 522 2205836, e-mail: nkumar_1650@yahoo.com

polyvinyl pyrrolidone. The homogenate was centrifuged at 15 000 *g* for 15 min. and used as crude enzyme. Chlase was assayed in 2 cm^3 reaction volume containing 100 mM phosphate buffer (pH 7.0), 0.24 % Triton X-100 and 0.2 µmol Chl *a* dissolved in acetone and 1 cm^3 of crude enzyme. The reaction was run for 15 min in dark at 40 ºC. Aliquots of 0.5 cm^3 were added to 5 cm^3 of phase separation mixture (acetone + hexane + 10 mM KOH; 2:3:0.2, v/v), vortexed for 30 - 40 s and the resulting emulsion was centrifuged at 12 000 g for 5 min for quick phase separation. Lower phase was withdrawn and chlorophyllide *a* estimated in the acetone phase at 667 nm using a coefficient of absorbance of 74.9 mM^{-1} cm^{-1}. One unit of activity was defined as the amount of enzyme hydrolyzing 1 nmol Chl a per s at 40 ºC. Protein in the enzyme extract was assayed according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

Chl contents were generally higher in female plants than in their male counterparts (Fig. 1). Chl contents in all the landraces showed decline with the onset of winter, however, the extent of change was less marked in female plants than in male plants. The process of recovery from low temperature commenced earlier in male plants as the chloroplast pigments started increasing from February, whereas in female plants this was delayed until March. Chl *a*/*b* also showed clear-cut differences between male and female landraces (Fig. 1). Based on this, the plants could be placed into two broad groups between 2.15 to 2.91 in female and 2.50 to 4.27 in male plants. Highest Chl *a*/*b* was observed in November and this transient change was independent of gender. The Chl *a*/*b* reverted back to earlier values later in December despite shortening of day length and fall in day and night temperatures. The changes in Cars were comparable to those in Chls. The contents of Cars in female plants did not change until January and declined only during subsequent months. In male plants, however, the trend was different as Cars showed decline due to cold stress followed by a rise once the environmental conditions became favourable. The ratio of Car/Chl was higher in males as compared to females (Fig. 1).

Chlase activity (Fig. 2) was low in October and increased with the onset of winters. The highest Chlase activity was observed in December. Though all the

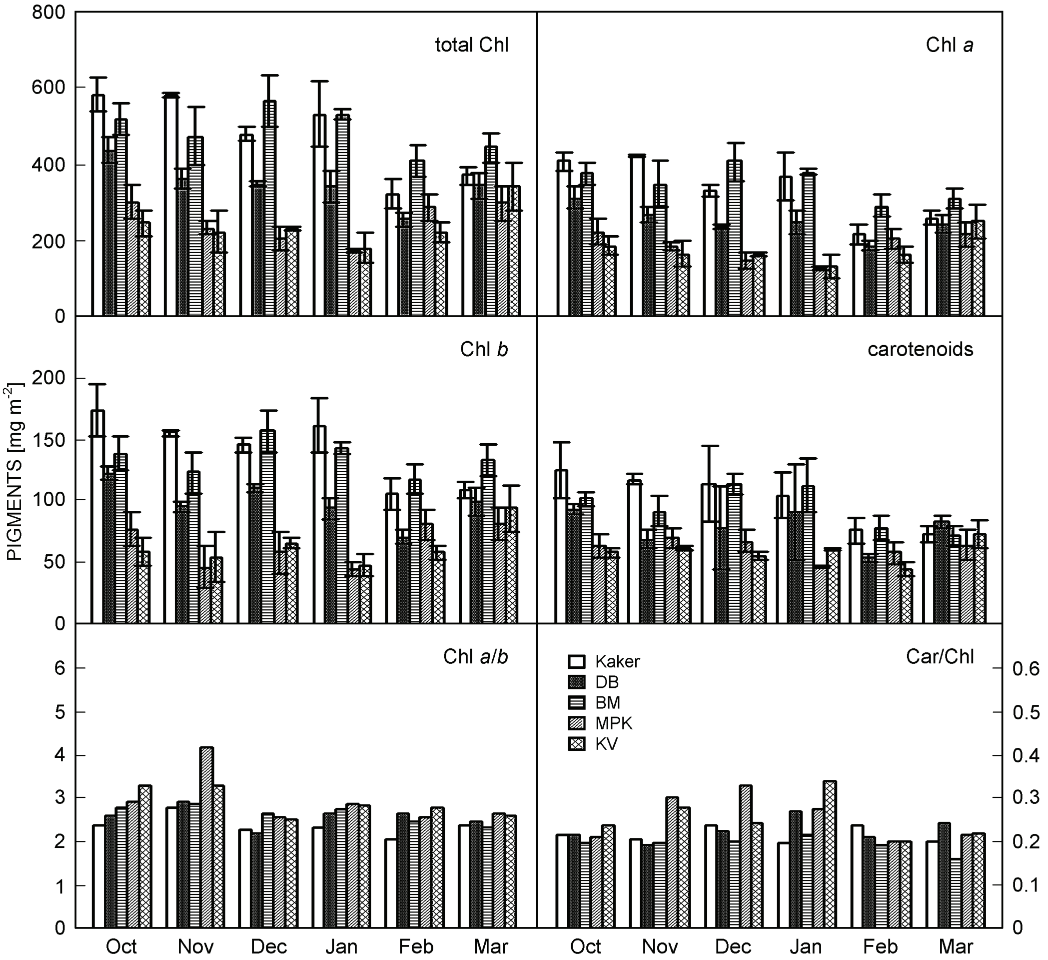

Fig. 1. Effect of low temperature stress on chlorophyll (Chl) and carotenoid (Car) contents in dioecious *Piper betle* male landraces Madras Pan Kapoori (MPK), Kapoori Vellaikodi (KV), and female landraces Kaker, Desi Bangla (DB) and Bangla Mahoba (BM). Pigment quantities are expressed on leaf area basis. Means ± SD, *n* = 6.

landraces showed this trend, it was more pronounced in male plants where activity remained higher until February. Considering the ratio of Chlase to Chl *a* content per leaf area unit, a similar pattern was evident, however, the differences between the landraces became sharper. In the females the ratio was <10 throughout the study with Kaker as an exception where was a transient change in December with a ratio of >10 which was still less than in male plants. This was perhaps due to appearance of dark brown spots on the leaf as a consequence of hypersensitive reaction to cold stress. In male plants, this ratio was always >10 and rose beyond 30 during winter. Thus the enzyme and its substrate ratio could serve as a good measure of plant response to stress.

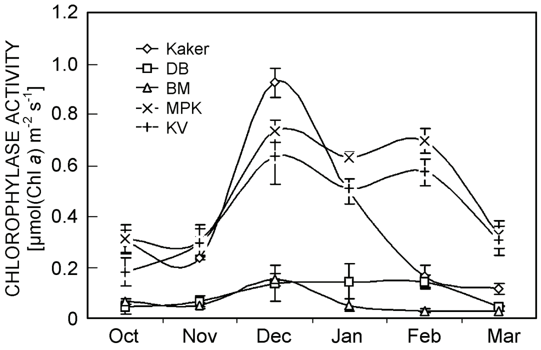

Fig. 2. Changes in chlorophyllase (Chlase) activity in male MPK and KV and female Kaker, DB and BM landraces of *P. betle* during different months. Means ± SD, $n = 6$.

Due to its tropical origin, *P. betle* is predisposed to low temperature stress encountered in most of the betel vine growing regions of North and Eastern India making it essential to grown it under fully protected conditions (Kumar 1999). The adverse effects of high temperature during summer were much less than those of low temperature during winter (data not shown). The Chl content in *P. betle* was high during active growth and declined with the onset of low temperature stress in December and January is in agreement with the observations on other plants (Lahiri and Singh 1969, Van Hasselt and Strikwerda 1976, Feierabend 1977, Tewari and Tripathy 1999, Tripathy *et al.* 2001). Chl breakdown is catalyzed by a set of enzymes and Chlase is the first in the sequence (Matile *et al.* 1989). Low temperature stress also altered the Chl *a*/*b* ratio signifying thereby the relative differences in biosynthesis and/or breakdown of Chl. However, the period of maximum change in Chl *a*/*b* ratio coincided with the onset of winter, which may cause slowed biosynthesis and increased degradation. The pigment composition of sun and shade plants have characteristic differences in the ratio of Chl *a*/*b*, which ranged from 2.5 to 2.9 in shade plants and 3.2 to 4.0 in sun plants (Lichtenthaler 1987). Despite plant growth at similar PAR in this study, the Chl *a*/*b* ratio differed in male and female landraces. Thus the differences in Chl *a*/*b* in *P. betle* may not be solely dependent on PAR apparently having also some relation with the phenomenon of dioecy. Higher Chl *a*/*b* in male plants could be due to increased rates of conversion of Chl *b* to Chl *a* through Chl *b* reductase (Folly and Engel 1999, Sheumann *et al.* 1999). The other possibilities could be lower stability of the membranes or fast turnover or both. In general, the Chl contents were inversely related with Chlase activity in *P. betle* in this study. This relationship suggests the involvement of Chlase in turnover of the macrocycle (Hendry and Stobart 1986, Drazkiewicz and Krupa 1991, Drazkiewicz 1994) and also in the breakdown of chlorophyll (Stoll 1912, Matile *et al.* 1999). Existence of Chlase in constitutive and inducible forms is known, *e.g.*, in *Citrus* and *Chenopodium* (Rise and Goldschmidt 1990, Shihoi *et al.* 1991, Trebitsh *et al.* 1993, Tsuchiya, *et al.* 1997). Induction of Chlase by ethylene and methyl jasmonate was reported in different plants (Trebitsh *et al.* 1993, Tsuchiya *et al.* 1999). Thus the activity of Chlase over and above constitutive level may be responsible for diverting the macrocycle to catabolism. Presence of two isoforms of Chlase were shown in *Chenopodium* (Tsuchiya *et al.* 1997) however, its functional significance has not been understood so far. The isoforms showed lower molecular mass compared to ethylene and jasmonate inducible Chlase from citrus peel (Trebitsh *et al.* 1993, Tsuchiya *et al.* 1999). Thus the enzymes reported from *Chenopodium* may be constitutive and the one from citrus peel may represent the inducible form. Our observations on changes in Chlase activity in response to low temperature stress also suggest the existence of constitutive and inducible form of Chlase in *P. betle*. Increased Chlase activity in degreening *Brassica napus* seeds due to sublethal freezing may be a case of low temperature induction (Johnson-Flanagan and McLachlan 1990). Our observations on rise of Chlase activity in *P. betle* due to low temperature stress; were also confirmed by independent experiments under controlled conditions (data not shown) are in agreement with these findings. Recently, Todorov *et al.* (2003) also showed the effect of low and high temperature and role of ethylene on Chlase in *Arabidopsis*.

Characteristic changes in Chlase activity in different landraces correlated with the amount of chloroplast pigments to the extent that any sharp changes in Chlase matched with the rise/decline in Chl contents. Chl turnover is fast in de-etiolating leaf (5 - 6 h) and slow in fully mature leaves where it takes more than 50 h (Stobart and Hendry 1984, Hendry and Stobart 1986). The decline in Chl content during low temperature stress is due to diversion of the macrocycle to catabolism as one of the adaptations to avoid the possible photoinhibition/ photodynamic damage during winter.

References

Dawson, T.E., Bliss, L.C.: Patterns of water use and tissue water relations in the dioecious shrub, *Salix artica*: the physiological basis for habitat partitioning between sexes. - Oecologia **79**: 332-343, 1989.

Dawson, T.E., Geber, M.A.: Dimorphism in physiology and morphology. - In: Geber, M.A., Dawson, T.E., Delph, L.F. (ed).: Gender and Sexual Dimorphism in Flowering Plants. Pp. 175-216. Springer-Verlag, Berlin - Heidelberg 1999.

Drążkiewicz, M.: Chlorophyllase: occurrence, function, mechanism of action, effects of external and internal factors. - Photosynthetica **30**: 321-331, 1994.

Drążkiewicz, M., Krupa, Z.: The participation of chlorophyllase in chlorophyll metabolism. - Acta Soc. Bot. Pol. **60**: 139-154, 1991.

Feierabend, J.: Capacity for chlorophyll synthesis in heat-bleached 70S ribosome-deficient rye leaves. - Planta **135**: 83-88, 1977.

Fernandez-Lopez, J.A., Almela, L., Almansa, M.S., Lopez-Roca, J.M.: Partial purification and properties of chlorophyllase from chlorotic *Citrus limon* leaves. - Phytochemistry **31**: 447-449, 1992.

Folly, P., Engel, N.: Chlorophyll *b* to chlorophyll *a* conversion precedes chlorophyll degradation in *Hordeum vulgare* L. - J. biol. Chem. **274**: 21811-21816, 1999.

Hendry, G.A.F., Stobart, A.K.: Chlorophyll turnover in greening barley. - Photochemistry **25**: 2735-2737, 1986.

Johnson-Flanagan, A.M., McLachlan, G.: The role of chlorophyllase in degreening canola (*Brassica napus*) seeds and its activation by sublethal freezing. - Physiol. Plant. **80**: 460-466, 1990.

Khalyfa, A., Kermasha, S., Marsot, P., Goetghebeur, M.: Purification and characterization of chlorophyllase from alga *Phaeodactylum tricornutum* by preparative native electrophoresis. - Appl. Biochem. Biotechnol. **53**: 11-27, 1995.

Kumar, N.: Betel vine (*Piper betle* L.) cultivation: a unique case of plant establishment under anthropogenically regulated microclimatic conditions. - Indian J. Hist. Sci. **33**: 19-32, 1999.

Lahiri, A.N., Singh, S.: Effects of hypothermia on the nitrogen metabolism of *Pennisetum typhoides*. - Proc. nat. Acad. Sci. India B **35**: 131-138, 1969.

Lichtenthaler, H.K.: Chlorophyll and carotenoids; pigments of photosynthetic biomembranes. - Methods Enzymol. **148**: 352-382, 1987.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Marshall, J.D., Dawson, T.E., Ehleringer, J.R.: Gender related differences in gas exhange are not related to host quality in the xylem-tapping mistletoe, *Phoradendron juniperinum* (Viscaceae). - Amer. J. Bot. **80**: 641-645, 1993.

Matile, P., Duggelin, T., Schellenberg, M., Rentsch, D., Bortlik, K., Peisker, C., Thomas, H.: How and why is chlorophyll broken down in senescent leaves? - Plant Physiol. Biochem. **27**: 595-604, 1989.

Matile, P., Hortensteiner, S., Thomas, H.: Chlorophyll degradation. - Annu. Rev. Plant Physiol. Plant. mol. Biol. **50**; 67-95, 1999.

Minguez-Mosquera, M.I., Gallardo-Guerrero, L: Role of chlorophyllase in chlorophyll metabolism in olives cv. Gordal. - Phytochemistry **41**: 691-697, 1996.

Nishiyama, Y., Kitamura, M., Tamura, S., Watanabe, T.: Purification and substrate specificity of chlorophyllase from *Chlorella regularis*. - Chem. Lett. **1**: 69-72, 1994.

Renner, S.S., Ricklefs, R.E.: Dioecy and its correlates in the flowering plants. - Amer. J. Bot. **82**: 596-606, 1995.

Rise, M., Goldschmidt, E.E.: Occurrence of chlorophyllides in developing light grown leaves of several species. - Plant Sci. **71**: 147-151, 1990.

Sheumann, V., Schoch, S., Rudiger, W.: Chlorophyll *b* reduction during senescence of barley seedlings. - Planta **209**: 364-370, 1999.

Shioi, Y., Tatsumi, Y., Shimokawa, K.: Enzymatic degradation of chlorophyll in *Chenopodium album*. - Plant Cell Physiol. **32**: 87-93, 1991.

Stobart, A.K., Hendry, G.A.F.: Chlorophyll turnover in greening wheat leaves - Photochemistry **23**: 27-30, 1984.

Stoll, A.: Über Chlorophyllase und die Chlorophyllide. - PhD. Thesis. ETH, Zürich 1912.

Tanaka, K., Kakuno, T., Yamashita, J., Horio, T.: Purification and properties of chlorophyllase from greened rye seedlings. - J. Biochem. **92**: 1763-1773, 1982.

Tewari, A.K., Tripathi, B.C.: Acclimation of biosynthetic reactions to temperature stress in cucumber (*Cucumis sativus* L.). - Planta **208**: 431-437, 1999.

Todorov, D.T., Karanov, N.E., Smith, A.R., Hall, M.A.: Chlorophyllase activity and chlorophyll content in wild type and eti 5 mutant of *Arabidopsis thaliana* subjected to low and high temperatures. - Biol. Plant. **46**: 633-636, 2003.

Trebitsh, T., Goldschmidt, E.E., Riov, J.: Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in citrus fruit peel. - Proc. nat. Acad. Sci. USA **90**: 9441-9445, 1993.

Tripathi, R., Khare, G., Kumar, N.: Effect of low temperature stress on *Piper betle* L. types, Bangla and Desavari. - J. Spices Aromatic Crops **9**: 141-143, 2000.

Tsuchiya, T., Ohta, H., Masuda, T., Mikami, B., Kita, N., Shioi, Y., Takamiya, K.-I.: Purification and characterization of two isozymes of chlorophyllase from mature leaves of *Chenopodium album*. - Plant Cell Physiol. **38**: 1026-1031, 1997.

Tsuchiya, T., Ohta, H., Okawa, K., Iwamatsu, A., Shiomada, H., Masuda, T., Takamiya, K.-I.: Cloning of chlorophyllase gene the key enzyme in chlorophyll degradation: finding of a lipase motif and induction by methyl jasmonate. - Proc. nat. Acad. Sci. USA **96**: 15362-15367, 1999.

Tyagi, R.K., Prakash, S.: Genotype-and sex-specific protocols for *in vitro* micropropagation and medium-term conservation of jojoba. - Biol. Plant. **48**: 19-23, 2004.

Van Hasselt, P.R., Strikwerda, J.T.: Pigment degradation in discs of the thermophilic *Cucumis sativus* as affected by light, temperature, sugar application and inhibitors. - Plant Physiol. **37**: 253-257, 1976.

Wang, X., Curtis, P.S.: Gender-specific responses of *Populus tremuloides* to atmospheric CO_2 enrichment. - New Phytol. **150**: 675-684, 2001.