Isolation and characterisation of gamma radiation-induced dwarf mutants of *Stylosanthes guianensis*

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Abstract

Stylosanthes guianensis is an important forage legume and cover crop in tropical and subtropical regions. Dwarf-type cultivars with enhanced chilling resistance would avoid competition for sunlight with young fruit trees when used as intercrop in orchards in subtropical regions. In this study, 7 dwarf mutants of *S. guianensis* were isolated from seedlings germinated following gamma ray irradiation of seeds of the cultivar, CIAT 184. The M4 and M5 plants were evaluated in 2006 and 2007, respectively. In greenhouse experiments, 2-month-old plants of the dwarf-mutant lines (4.2-2, 4.2-3, 4.2-4, 4.2-6, 4.2-11 and 7-2) were 31–34% shorter than the wild type control (CIAT 184). However, the dry weights of shoots and roots of the mutant lines were generally similar to those of the wild type control (P>0.05), while the shoot dry weight of line 4.2–3 was significantly greater than for CIAT 184 (P<0.05). Growth of the dwarf mutants was promoted by treatment with 50 mg/L and 100 mg/L gibberellin (GA_3), while CIAT 184 was not affected, indicating that GA_3 biosynthesis might be blocked in the mutants. All mutant lines had increased drought tolerance over that of the parent, and the lines 4.2-4, 4.2-6 and 4.2-11 had increased chilling resistance. In field tests, the tested mutant lines (4.2-3, 4.2-4, 4.2-6, 4.2-11 and 7-2) were 33% and 26% shorter than their parent cultivar, CIAT 184, in 2006 and 2007, respectively. The chlorophyll concentrations in leaves were also higher in the mutant lines than in CIAT 184.

Introduction

Stylosanthes guianensis is an important forage legume with high yield and quality, tolerance of soil acidity and excellent adaptation to infertile soils in tropical and subtropical countries (Meijer and Broughton 1981; Guodao *et al.* 1997; Miles and Grof 1997; Miles and Lascano 1997). It is used as a cover crop in plantation agriculture, as green manure, as a source of fresh fodder or hay and to increase soil fertility for mine-site rehabilitation (Grigg *et al.* 2000; de la Rue *et al.* 2003). As an excellent tropical forage legume and cover crop in fruit-tree and rubber plantations, *S. guianensis* has been widely cultivated in southern China since its introduction from South America and Australia (Jiang *et al.* 2005). In recent years, it has been used to stabilise soils along highways in southern China. However, low temperatures in winter limit its growth and production in subtropical regions, as photosynthesis is reduced during chilling stress (Zhou *et al.* 2006). The plant hormone abscisic acid (ABA) increases its chilling tolerance through induction of an antioxidant defence system (Zhou *et al.* 2005a), which is initiated by the signal molecule nitric oxide (NO) (Zhou *et al.* 2005b) and Ca^{2+} (Zhou *et al.* unpublished data). Investigations of chilling tolerance of *S. guianensis* or breeding for chilling-tolerant cultivars are limited. Dwarf-type cultivars with enhanced tolerance of cold temperatures should avoid competition for soil nutrients and sunlight with young fruit trees and increase amenity and sustainability when planted on roadsides or as intercrop in orchards.

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Gamma ray irradiation is an efficient tool to produce mutants in crop breeding and more than 1800 cultivars either obtained as direct mutants or derived from their crosses have been released worldwide in 50 countries (Ahloowalia and Maluszynski 2001). Large-seeded mutant breeding lines have been isolated from the gamma irradiation-induced 'Georgia Browne' peanut cultivar (Branch 2002). Seedless and *Mal Secco*-tolerant mutants of lemon have been selected through gamma ray irradiation (Gulsen *et al*. 2007). Irradiation-induced mutations have also succeeded in forage grasses and turfgrasses. Non-arrowing and broad-leaved mutants of rhodes grass with increased yield and crude protein, nitrogen and calcium accumulation have been isolated following gamma irradiation (Krishna *et al*. 1984). Bermuda grass cultivars of 'TifEagle', 'Tift 94' and 'Tifway II' were selected from gamma-irradiated mutant lines (Burton 1985; Hanna *et al*. 1997; Hanna and Elsner 1999). Gamma ray irradiation has also been used in the breeding of centipede grass and St Augustine grass (Busey 1980; Dickens *et al*. 1981). By use of gamma irradiation, a disease-resistant cultivar of *S. guianensis* has been released and used in southern China (Liang *et al*. 1998).

In order to induce and select chilling-tolerant mutants of *S. guianensis*, seeds were irradiated with gamma rays and 10 dwarf mutants with increased stress tolerance were selected. This paper reports the induction, isolation and characterisation of the dwarf mutants of *S. guianensis* induced with gamma ray irradiation.

Materials and methods

Plant materials and mutation treatment

Seven groups of seeds of *Stylosanthes guianensis* cv. CIAT 184 were packaged in envelopes and irradiated with gamma rays at dosages of 400, 420, 440, 460, 500, 600 and 700 Gy (for 12 000, 7 000, 16 000, 7 000, 12 000, 1 300 and 3 000 seeds, respectively) using a ^{60}Co source at the Irradiation Centre, South China Agricultural University in April 2003. As we expected to select mutants rather than to establish a standard protocol for gamma ray irradiation, and the irradiation was not carried out at the same time, no identical seeds in total were irradiated in the experiments. The irradiated seeds plus untreated seeds (control) were then soaked in hot (80°C) water for 5 min, rapidly cooled to room temperature and soaked again in cold water overnight. The seeds were sown in 15 cm diameter plastic pots containing a mixture of peat and perlite (3:1, v/v) to encourage germination. Fifteen to 20 plants were kept in a single pot. The seedlings were grown under natural light in a greenhouse at a temperature of 25–30°C. Plants were irrigated daily, and fertilised weekly by irrigating with 100 ml of a 0.3% solution of N:P:K fertiliser (15:15:15) for 2 months.

Selection of dwarf-type mutant lines

The 2-month-old M1 seedlings were grown in growth chambers illuminated at 800 µmol/m^2/s at 3°C with 12 h light for 3 days, followed by a recovery period at room temperature (25–30°C) for 3 days in June 2003. From the surviving plants, 10 dwarf-type plants were selected. These plants were individually grown in a greenhouse for self-pollination to harvest M2 seeds in December 2003. The procedure was repeated in 2004 and 2005 to harvest M3 and M4 seeds, respectively. The M4 seeds were planted and M5 seeds were harvested in the field in 2006. M4 and M5 plants were evaluated in 2006 and 2007, respectively.

Determinations of plant weight and height

M5 seeds of the isolated mutants plus the control were germinated in 15 cm diameter plastic pots containing a mixture of peat and perlite (3:1, v/v), with 5 replications for each line. Seedlings were grown under natural light in a greenhouse at temperatures of 25–30°C. Plants were irrigated daily, and fertilised weekly by irrigating with a 0.3% solution of N:P:K fertiliser (15:15:15). For measurements of weight of shoots and roots of plant, some of the 2-week-old seedlings of a similar size were transplanted to new pots with one plant per pot and were grown for an additional 6 weeks. Shoots and roots were separated and dried at 80°C overnight for measurements of weight of shoots and roots. For measurement of growth as affected by gibberellin (GA$_3$), 6-week-old seedlings were grouped into 3 and allocated to the following treatments: (1) water; (2) 50 mg/L GA$_3$; and (3) 100 mg/L GA$_3$. These solutions, containing 0.05% Tween-20,

were sprayed uniformly on the plants until the leaf surfaces were thoroughly wet. There were 7 lines and 3 treatments with 5 pots as replications for each treatment and plants were placed randomly. Plant height was measured after 2 weeks.

Measurements for chilling and drought tolerance

Ten M5 seedlings were planted in 15 cm pots and grown in a greenhouse for 2 months as described above. Seven lines with 5 pots per line were subjected to chilling treatment at 6°C in a growth chamber for 5 days. To simulate dry conditions, water was withheld from another group of plants for 4 days. Relative water content (RWC), ion leakage and maximal photochemical efficiency of photosystem II (F_v/F_m) of the second leaflets from the top were measured as described by Zhou *et al.* (2005a; 2006). RWC and ion leakage were used to assess drought tolerance, while ion leakage and F_v/F_m were used for evaluation of chilling resistance.

Field tests

M4 and M5 plants were tested in the field of South China Agricultural University. Two-month-old M4 seedlings were transplanted into the field on June 30 and plant height was measured on September 30, 2006. In 2007, two-month-old M5 seedlings were transplanted into the field on May 10 with 3 plots (3 × 4 m)/line and 30 plants/plot. Plant height was measured on September 19, 2007. The soil contained 0.49 g/kg available N, 5.1 mg/kg available P, 89.2 mg/kg available K and 1.08% organic matter and had pH 5.7.

Chlorophyll comcentration in the third fully expanded leaf (0.5 g) was determined as described by Arnon (1949) on October 29, 2006 (M4) and September 25, 2007 (M5). The leaves were ground with a mortar and pestle, and extracted with 10 ml of 80% ethanol for 1 h. After centrifugation, the absorbance of the supernatants was read at 663 nm and 645 nm.

Statistical analysis

All treatments were arranged in a completely randomised design with 3–5 replicates. All data in greenhouse and field tests were statistically analysed by 2-way ANOVA using the generalised linear model procedure in the SAS statistical program.

Results

Selection of dwarf-mutant lines

Following irradiation in April 2003, an average of 40% of tested seeds germinated. Ten dwarf-type plants were observed, 6 from the 420 Gy gamma-irradiated seeds, which were named 4.2-2, 4.2-3, 4.2-4, 4.2-6, 4.2-7 and 4.2-11, respectively, while a single dwarf plant from the 700 Gy gamma-irradiated seeds was named 7-2.

During 2004 and 2005, all lines of M2 and M3 plants showed dwarfism, and no segregation was observed. Since 2006, the morphological characters of 6 mutant lines (4.2-2, 4.2-3, 4.2-4, 4.2-6, 4.2-11 and 7-2) plus the wild type control (cultivar CIAT 184) were compared annually in the greenhouse and fields. Two-week-old M5 plants were transplanted into pots and grown for an additional 6 weeks in the greenhouse, when plant height and dry weights of shoots and roots were measured. The data for 2007 are presented in Figures 1A and 1B. All mutant lines (4.2-2, 4.2-3, 4.2-4, 4.2-6, 4.2-11 and 7-2) were 31-34 % shorter than the control (Figure 1A). However, the dry weights of shoots and roots of the mutant lines were generally similar to those of the control, although line 4.2-3 had higher (P<0.05) shoot dry weight than CIAT 184 (Figure 1B).

Plant height and responses to plant growth regulators

Growth of the control (CIAT 184) was unaffected by treatment with GA_3 (Figure 2). However, treatment with GA_3 promoted growth of all dwarf-mutant lines, with lines 4.2-2 and 4.2-11 showing increasing responses to GA_3 up to the higher level, 100 mg/L.

Resistance to chilling and drought stress

F_v/F_m was maintained at about 0.85 in all the tested plants before chilling treatment. After seedlings were subjected to chilling at 6°C in a growth chamber for 5 days, F_v/F_m decreased
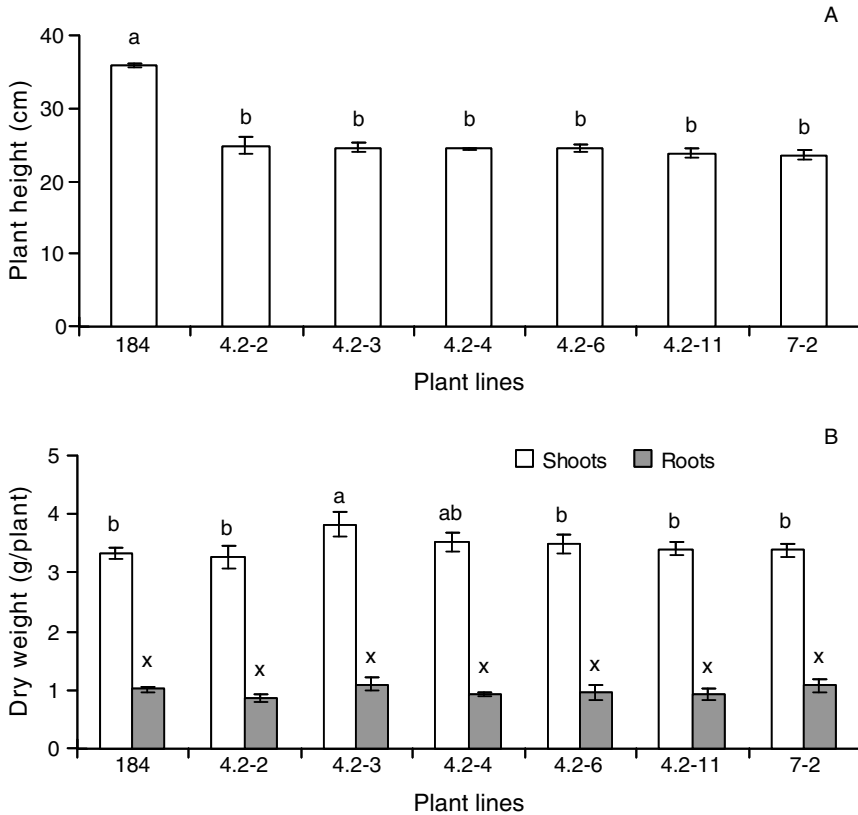
Figure 1. Plant height (A) and dry weight of shoots and roots (B) of the dwarf-mutant lines and the parent cultivar (CIAT 184) at 8 weeks of age in a greenhouse. Vertical bars reprent s.e. of mean. Within parameters, columns with the same letter were not significantly different (P = 0.05).

significantly to 0.45 in the control, but was 0.57, 0.61 and 0.67 in the mutant lines 4.2-4, 4.2-6 and 4.2-11, respectively, 27–48% higher than in the control. After a recovery period at room temperature, F_v/F_m increased in all mutant lines, but not in the control (Figure 3A). Ion leakage was about 6–8% in the tested plants before chilling (data not shown). Values increased to 31% in the control after chilling, but were 25, 26 and 23% in the mutant lines 4.2-4, 4.2-6 and 4.2-11, 15–27% lower than in the wild type control. After a recovery period at room temperature, ion leakage in all plants increased, but at a relatively lower level in mutants 4.2-4, 4.2-6 and 4.2-11. Although line 7-2 had lower ion leakage after chilling treatment than the control, its ion leakage increased to a level similar to that of the control after recovery (Figure 3B).

Relative water content was 93–95% in the tested plants under normal growth conditions

(data not shown). While withholding irrigation for 5 days caused wilting in all plants, the mutant lines had significantly (P<0.05) higher RWC and lower ion leakage than the control. Rewatering resulted in the recovery of RWC in the mutant lines, while the control remained wilted (Figure 4A). Although rewatering increased ion leakage in all plants, ion leakage in the dwarf-mutant lines remained significantly lower than in the control (Figure 4B).

Morphological characteristics of the dwarf-mutant lines in the field

Height of control plants in September 2006 was 62.7 cm, compared with a mean of 41.2 cm in the dwarf-mutant lines (4.2-3, 4.2-4, 4.2-6, 4.2-11 and 7-2) (Figure 5A). In September 2007, the comparable values were 67.5 cm and
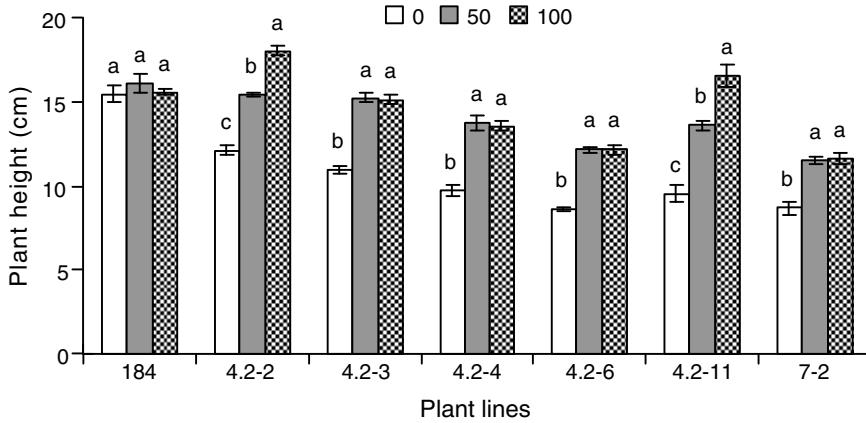
Figure 2. Plant height of the dwarf-mutant lines and the parent cultivar (CIAT 184) after treatment with 50 and 100 mg/L of gibberellin (GA_3) or water as control for 2 weeks. Vertical bars represent s.e. of mean. Within lines, columns with a common letter were not significantly different (P = 0.05).
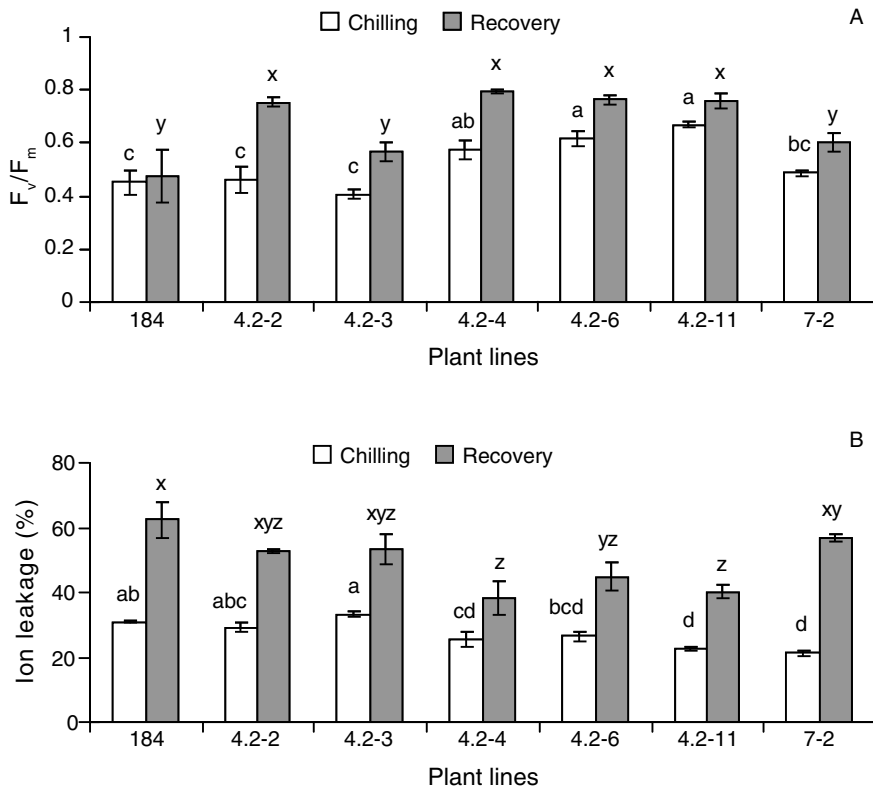

Figure 3. F_v/F_m (A) and ion leakage (B) of the dwarf-mutant lines and the parent cultivar (CIAT 184) grown at low temperature (6°C) for 5 days, followed by recovery for 2 days at room temperature. Vertical bars represent s.e. of mean. Columns with a common letter at a given measurement from each treatment were not significantly different (P = 0.05).
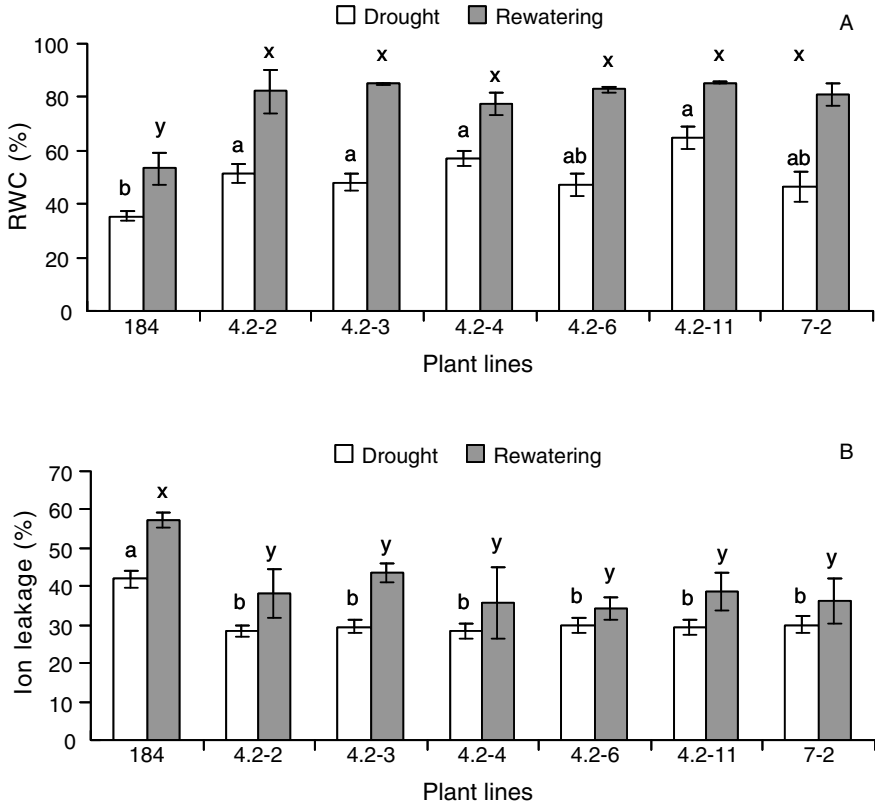
Figure 4. Relative water content (RWC, A) and ion leakage (B) of the dwarf-mutant lines and the parent cultivar (CIAT 184) after withholding irrigation for 5 days, followed by recovery of re-irrigation for 2 days. Vertical bars represent s.e. of mean. Columns with a common letter at a given measurement from each treatment were not significantly different (P = 0.05).

50.0 cm for the control and mean of mutant lines, respectively.

The leaves of the mutant lines were greener than controls in the field. Chlorophyll concentrations in mutant lines were similar and were 86–94% and 14–23% higher than in the control in 2006 and 2007, respectively (Figure 5B). Differences in chlorophyll concentration in the dwarf-mutants between 2006 and 2007 occurred. We have no satisfactory explanation for this phenomenon.

Discussion

This study has demonstrated that gamma ray irradiation of seeds of *S. guianensis* can produce dwarf-mutant lines, with greater drought tolerance and chilling resistance than the original par-

ents. Ten mutant lines of *S. guianensis* exhibited dwarf characteristics and 6 lines were selected with shorter plant height and higher chlorophyll concentration in leaves than the wild type control. Since the dwarf trait remained stable through 5 generations (M1–M5), this study supports earlier findings from use of gamma irradiation in the development of dwarf Bermuda grass cultivars (Burton 1985; Hanna *et al.* 1997; Hanna and Elsner 1999; Lu *et al.* 2008) and St Augustine cultivars (Busey 1980), that irradiation mutation is a feasible means to generate morphologically diverse germplasm.

Burton (1985), Hanna *et al.* (1997) and Hanna and Elsner (1999) suggested that the slow-growing trait might lead to low water use, making them suited to a greater range of rainfall conditions. The increased drought tolerance of our dwarf mutants in comparison with the parent control,
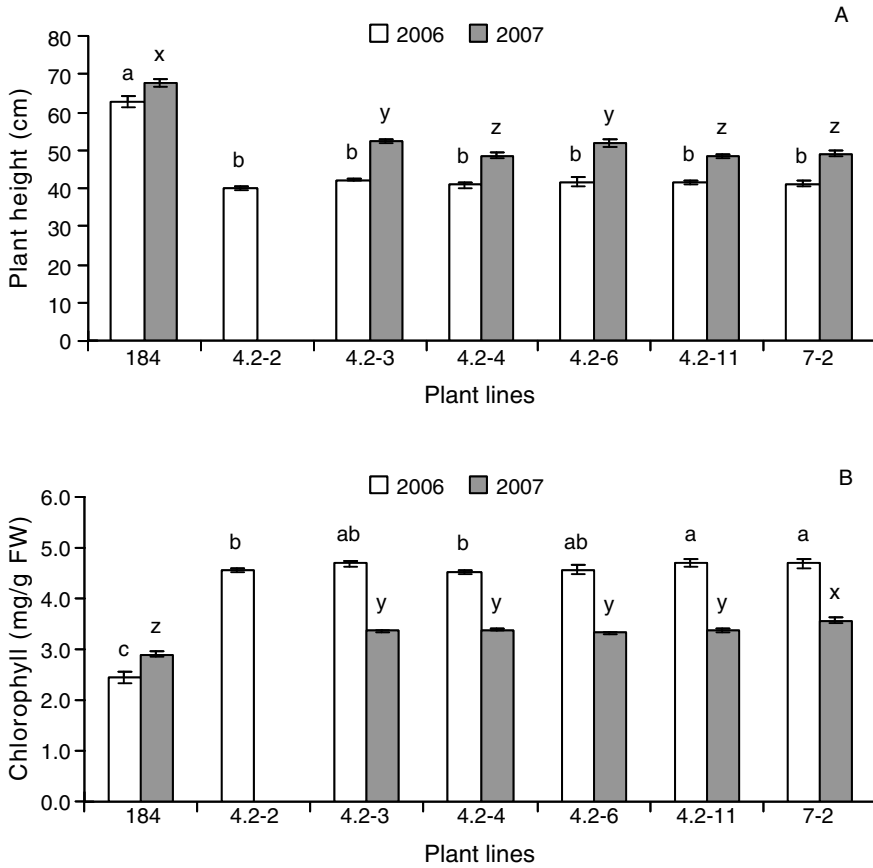
Figure 5. Plant height (A) and chlorophyll concentrations in leaves (B) of the dwarf-mutant lines and the parent cultivar (CIAT 184) in the field experiments in 2006 and 2007. Vertical bars represent s.e. of mean. Columns with a common letter at a given measurement from each year were not significantly different (P = 0.05). The line 4.2-2 was not tested in 2007.

as manifested by their maintenance of higher RWC and lower ion leakage under drought stress in the greenhouse tests, supports this hypothesis. RWC and ion leakage are widely used as physiological indices of drought resistance in plants (Guo *et al*. 2006), as RWC reflects water status in plants, while ion leakage reflects the membrane damage under stress conditions. Recent studies with bentgrass species indicated that slow-growing turfgrass can survive under drought stress for prolonged periods in response to reduced water use and increased osmotic adjustment and antioxidant activity (DaCosta and Huang 2006; 2007).

The increased chilling resistance of 3 mutant lines, as manifested by their maintenance of higher F_v/F_m and lower ion leakage under chilling stress in the growth chamber, suggests these lines will be adapted to much colder environments than the parent line. This trait, combined with the enhanced resistance to drought, would widen their range of environmental adaptation and practical use, while the higher chlorophyll content should result in greater photosynthesis and carbohydrate production, producing better yields. The dwarf-mutant lines, especially 4.2-4, 4.2-6 and 4.2-11, with enhanced drought and chilling resistance, should be evaluated in southern China as cover crops, as intercover plants in fruit-tree and rubber plantations for improving soil fertility and as a forage source.

Although gibberellin (GA_3) stimulates internode elongation of plants (Ross *et al*. 1997), dose rates of 50 and 100 mg/L of GA_3 had no effect on the height of the control plants. However, the increased growth of all mutant lines in response to GA_3 suggests that the mutant lines might have

lower GA$_3$ levels *in vivo* than the parents. Other studies (Chandler and Robertson 1994; Lu *et al.* 2003; Zhou *et al.* 2005a) suggest that reduced GA$_3$ biosynthesis in the dwarf mutants might improve ABA levels in the plants, resulting in increased drought tolerance and chilling resistance. Evidence exists that genetic manipulation of one biosynthesis pathway can moderate the activity of a second pathway (Batge *et al.* 1999; Fray *et al.* 1995; Fraser *et al.* 1995). This explanation of altered flux relies on the assumption that the biosynthetic pathways for ABA and GA$_3$ share the same precursor, geranylgeranyl diphosphate. An analogous situation might exist in the dwarf-mutant lines. The relationship of GA$_3$ and ABA levels in the dwarf-mutant lines in comparison with the parent will be studied in the future to elucidate the mechanisms through which the dwarf-mutant lines have enhanced drought tolerance. Understanding the mechanisms that are operating might make it possible to utilise this strategy in developing better adapted plants for other stressful situations.

Acknowledgements

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