

## COMPARISON OF THE FROST RESISTANCE OF BARLEY (*HORDEUM VULGARE* L.) LANDRACES OF UPLAND ETHIOPIA USING ELECTROLYTE-LEAKAGE AND CHLOROPHYLL FLUORESCENCE

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**ABSTRACT:** Barley is the most important crop in the highlands of Ethiopia at altitudes above 2600 m, where its productivity is limited by cold stress. We studied 25 Ethiopian barley landraces in order to identify cold tolerant types and to describe characteristics and acclimation potentials of these landraces to cold stress. Barley plants were grown from seeds in a temperature-controlled greenhouse (20/15°C) for six weeks prior to investigation. Membrane leakage, assayed by electric conductivity, was investigated before and after the plants had been subjected to freeze-thaw cycles at different sub-freezing temperatures down to -10°C. The critical temperature (CT<sub>50</sub>), at which the frost-induced damage was 50% of the maximal injury, ranged for most landraces between -5.6 and -8.6°C. Maximum difference in sensitivity to frost among the various races was observed at -5.0°C. Neither the altitude of the habitat, from where the seeds had been collected, nor the maturation time of the landraces were correlated with the degree of frost tolerance of the landraces. The influence of the growth conditions on the capability of frost hardening of cold-tolerant and sensitive landraces was investigated using chlorophyll *a* fluorescence. Barley seedlings were grown under three different regimes: In a temperature controlled greenhouse (20/15°C, day/night), under simulated tropical alpine conditions (tropical-alpine greenhouse, 16/0°C, 12 h light period), and in a phytotrone at constant 2°C day and night. Effective quantum yield of photosynthesis was measured at several temperatures in the course of freeze-thaw cycles (+20 to -10 to +20°C). Whereas growth and the effective photosynthetic quantum yield of the plants in the phytotrone were low, cold hardening was effective as shown by the recovery of the photosynthetic quantum yield upon re-warming of the frozen plants. Effective quantum yield of the plants grown in the tropical alpine greenhouse was as high as with the control plants, but only 2 of 6 investigated landraces showed immediate recovery of the photosynthetic quantum yield after freezing. Landraces which showed the highest cold tolerance were found to acclimatize best.

**Key words/phrases:** Barley, chlorophyll fluorescence, cold acclimation, Ethiopia, frost tolerance

### INTRODUCTION

Barley (*Hordeum vulgare* L.) is a traditional crop in the highlands of Ethiopia, where it has been cultivated for at least 5000 years (Purseglove, 1972). It is the major staple food grain utilised in various forms of foodstuff, which accounts for about 60% of the highland population's total plant food (Zemedede Asfaw, 1990). Ethiopian barley germplasm is of considerable importance to barley breeding world-wide having such characteristics as distinct grain colour, high lysine content, high tillering capacity, but also a fragile rachis (Berhane Lakew *et al.*, 1996). In many

African tropical high mountains, where barley is usually the only crop grown at altitudes between 3000 and 4000 m, mild to moderate nocturnal frost is a component of every day's weather, especially in the dry seasons (Hedberg, 1964). In these regions, diurnal temperature fluctuations could vary from nocturnal temperatures of -8°C to day-time temperatures of +15°C (Masresha Fetene *et al.*, 1998). Field observations in various highlands of Ethiopia have indicated that frost is a major constraint of barley production, sometimes causing complete crop failure (Asmare Yallew *et al.*, 1998; Chilot Yirga *et al.*, 1998). Most research into responses of barley to frost has

focused on summer or winter barley of temperate provenances (see *e.g.*, Hansen and Beck, 1988). There are neither reports on effects of frost on barley in the tropics, nor have there been efforts to scientifically identify frost resistant landraces. The present work is an attempt to assess the issue of frost tolerance of Ethiopian barley landraces collected from different areas and altitudes.

To discriminate between frost tolerant and frost-sensitive plants, various methods have been used: measurement of electrical conductivity (Barranco *et al.*, 2005), vital staining (Fiorino and Mancuso, 2000) and photosynthetic efficacy (Bodner and Beck, 1987). Recently, chlorophyll *a* fluorescence is increasingly being used for evaluation of frost tolerance as a rapid, precise and non-invasive method capable of monitoring a trait related to a crucial stage in the acquisition of frost tolerance (Rizza *et al.*, 2001; Agueda *et al.*, 2005; Neuner and Pramsöhler, 2006). In the present study we used electrolyte leakage and chlorophyll *a* fluorescence to evaluate frost tolerance in barley landraces of Ethiopia. In detail, the investigation addresses the following questions: (1) how do landraces rank in terms of frost tolerance; (2) is the degree of cold hardiness correlated with the altitude at which the landraces are grown; (3) is cold hardiness correlated with the time period required for maturation; and (4) to what degree can barley landraces acclimatize to subfreezing temperatures.

## MATERIALS AND METHODS

### *Collection of plant material*

Samples were collected from known barley growing areas of Ethiopia, which are listed in Table 1. The altitudes of these areas range between 2300 and 3630 masl. Sampling of the landraces was made non-selectively at random by meandering along contours from one end to the other of the selected crop fields taking an ear at each foot step. About 50 samples were taken from each farmland that confirmed the probability of 95% sampling (Marshall and Brown, 1975).

Samples from altitudes outside the surveyed areas (below 2900 m and above 3500 m) were obtained from the Institute of Biodiversity Conservation in Addis Ababa (IBC). In total 25 landraces were investigated (Table 1).

### *Growth conditions*

Seeds from a single ear of each sample were germinated in Petri dishes (12.5 cm in diameter) on moist filter paper for about 5 days. Three seedlings, about 5 cm long, were planted into plastic pots filled with 2.5 kg garden soil and grown in a temperature-controlled greenhouse at 20/15°C day/night temperature, 55% rh, and a light period of 12 h in natural day light supplemented with high pressure sodium lamps providing a minimum photon flux density of 400  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Of each landrace, a total of ten pots with three seedlings each, were cultivated. For determination of frost resistance, six-week-old plants were used. For the cold-acclimation studies three week old seedlings were transferred either to a special greenhouse, that simulates the tropical alpine environment or to a temperature- and light controlled phytotrone. Under all three growth conditions (common greenhouse as control, tropical alpine greenhouse and phytotrone) the daily light period was strictly 12 hours. The temperature regime in the tropical-alpine greenhouse was 12/0°C day/night with up to 85% rh during the night and 50–60% during the day. The temperature in the phytotrone was maintained constant at 2°C. All plants were regularly watered.

### *Analysis of cold hardiness: The freeze-thaw cycle*

Cold hardiness was examined after subjecting the leaves to a freeze-thaw cycle. Leaves were placed in test tubes with the lower end immersed in 0.5 ml water. The tube was sealed and transferred into a small chamber which was cooled by a refrigerated bath containing ethylene glycol as the coolant. The chamber was allowed to cool to the desired temperatures (0–10°C) at the rate of -1°C per hour using a datalogger as control unit.

Table 1. Landraces used in the study.

No	Landrace number	Local name of variety	Area of collection			Altitude (masl)
			Province/Zone	Awraja/District	Place name	
1	AC3268*	Garboo	Bale	Wabe	Ginir	2300
2	AC3267*	Chenka	Bale	Wabe	Ginir	2300
3	AC3235*	Belga-Gebs	Gonder	Gonder	Gonderzuria	2350
4	AC1696*	Senef-kollo	Arsi	Ticho	Tena	2400
5	AC3260*	Kokufa	Bale	Mendeyo	Agarfa	2430
6	AC3231*	Menso	Gojjam	Agewmidir	Banja	2460
7	AC3232*	Wenteka	Gojam	Agewmidir	Banja	2460
8	AC1759*	Balemi	Shewa	Jibat and Mecha	Dondi	2870
9	C-030	Zewtat	East Tigray	Ganta-Afeshum	Mitsnah	2900
10	C-034	Saesaa	East Tigray	Ganta-Afeshum	Mitsnah	2900
11	C-035	Gunanzsigem	East Tigray	Ganta-Afeshum	Mitsnah	2990
12	C-028	Tsaeda-Atena	East Tigray	Ganta-Afeshum	Mitsnah	3000
13	C-018	Keyih-shewa	South Tigray	Enda-Mehoni	Tsibet	3000
14	C-019	Haftu-sene	South Tigray	Enda-Mehoni	Tisbet	3000
15	C-021	Tsaeda-shewa	South Tigray	Enda-Mehoni	Tisbet	3100
16	C-025	Tselm-sigem	South Tigray	Enda-Mehoni	Tisbet	3130
17	C-023	Tselim	South Tigray	Enda-Mehoni	Tisbit	3150
18	AC64091*	-	Arsi	Kefeke	-	3200
19	C-013	Sekutere	North Wollo	Bugna	Abune Yosef	3270
20	C-003	Sekutere	North Wollo	Bugna	Abune Yosef	3300
21	AC4839*	-	Wollo	Werehimono	Legambo	3500
22	AC235079*	Gebs	Wollo	Werehimono	Legambo	3500
23	AC4941*	Tikur-Gebs	Wollo	Wereilu	-	3550
24	AC235096*	Gebs	Shewa	Menz and Gishe	-	3600
25	C-012	Nechita	NorthWollo	Bugna	Abune Yosef	3630

Note: Landraces with asterisks have been obtained from the Institute of Biodiversity Conservation, Addis Ababa.

The desired final subfreezing temperature was maintained for one hour, after which the test tubes were placed in an ice chest filled with crushed ice in water allowing slow re-warming overnight. Damage by the subfreezing temperatures was determined using the electrical conductivity method as described by Palta *et al.* (1977). The extent of membrane damage was determined as the percentage of maximum possible injury, which was produced by autoclaving of the samples (Flint *et al.*, 1967; Janacek and Prasil, 1991).

Frost hardiness of the examined 25 barley landraces was characterized by the critical temperature  $CT_{50}$ .  $CT_{50}$  is the subzero temperature at which ion leakage was 50% of the maximum, a point interpreted to correspond to irreversible lethal damage (Janacek and Prasil, 1991; Boorse *et al.*, 1998). To derive the  $CT_{50}$  temperatures by interpolation, logistic curves were fitted to the conductivity data.

#### Chlorophyll fluorescence

Chlorophyll *a* fluorescence of leaves of barely landraces grown under the different conditions described above was determined with a portable

pulse-amplitude modulated photosynthesis yield analyzer (Mini-PAM, Heinz Walz, GmbH, Effeltrich, Germany) equipped with a standard 2030-B leaf clip holder. The efficiency of PSII photochemistry ( $F_m - F_o$ )/ $F_m = F_v/F_m$  was measured at predawn with pre-darkened leaves, where  $F_o$  is the antenna fluorescence of dark adapted leaves,  $F_m$  is the maximal fluorescence level with all reaction centres closed after a saturating light pulse ( $3000 \mu\text{mol m}^{-2}\text{s}^{-1}$  on the dark adapted leaves) and  $F_v$  is the maximal variable fluorescence. Effective quantum yield of PSII was calculated as  $(F_m' - F)/F_m' = \Delta F/F_m'$ , (Genty *et al.*, 1989; Masresha Fetene *et al.*, 1997), where  $F$  is the steady-state value of fluorescence of the light adapted leaf and  $F_m'$  is the maximal fluorescence level of the light adapted state determined by saturating light pulses ( $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) superimposed on the usual actinic light flux density.

#### Cold-acclimation and chlorophyll fluorescence measurements at subfreezing temperatures

Samples from plants grown in the greenhouse (control), in the tropical-alpine greenhouse or in the phytotrone, were used for investigating the

potential for cold acclimation and recovery from a freezing event. Chlorophyll *a* fluorescence was used as parameter. Maximum quantum yield of PSII was determined at various temperatures in a freeze-thaw cycle. Leaf portions of 2 cm length, taken from the centre of the leaves were put in a special glass chamber (Elub 0601, Cooling Chamber, produced by the workshops of the University of Bayreuth, Germany) which could be cooled or warmed by a Peltier element attached to its base. The Peltier element was connected to a control unit whose output was recorded with a datalogger. The leaf was illuminated with the light-pipe of the Mini-PAM fluorometer which was fixed in a hold in the lid of the chamber. The cooling treatment of the leaves consisted of a freeze-thaw cycle, starting at 20°C with a cooling and re-warming rate, respectively, of 2°C/h. Measurements of the chlorophyll *a* fluorescence parameters were taken at +20, +10, +2.5, 0, -2.5, -5.0, -7.5, -10, -7.5, -5.0, -2.5, 0, +2.5, +10 and +20°C.

### Statistical analysis

All data are mean values of at least three individual measurements with the standard errors. In Figure 5, where up to 6 curves overlap, standard errors have been omitted for the sake of clarity of the plots. Logistic curves were fitted to the conductivity data, to derive the  $CT_{50}$  temperatures by interpolation using SAS (1990).

## RESULTS

### Critical temperatures ( $CT_{50}$ ) and comparison of the responses of landraces subjected to cooling at -5°C

Using mature leaf blades of the individual landraces the subzero temperatures were determined after a freeze-thaw cycle at which 50% of the maximum ion leakage ( $CT_{50}$ ) occurred (Fig. 1). This is the degree of membrane damage which marks the onset of irreversible lethal damage (Flint *et al.*, 1967; Boorse *et al.*, 1998). For most landraces  $CT_{50}$  ranged between -5.6 and -8.8°C. The most frost tolerant landraces were C-003, C-012, C-018, AC3260, and AC3267 with  $CT_{50}$  between -8.5 and -8.8°C. The best way to show the individual differences of frost tolerance was to compare the degree of damage (as measured by ion leakage) incurred with a minimum temperature of -5°C (Fig. 2). At that non-lethal

subfreezing temperature, ion leakage varied between 5 and 30%. Samples that showed relative frost tolerance with less than 10% damage were C-003, C-013, C-018, AC3260, AC3235, and AC3268. The most sensitive samples at -5°C were C-021, C-019, AC64091, AC235096, C-034 and AC1759 which were damaged by more than 20%.

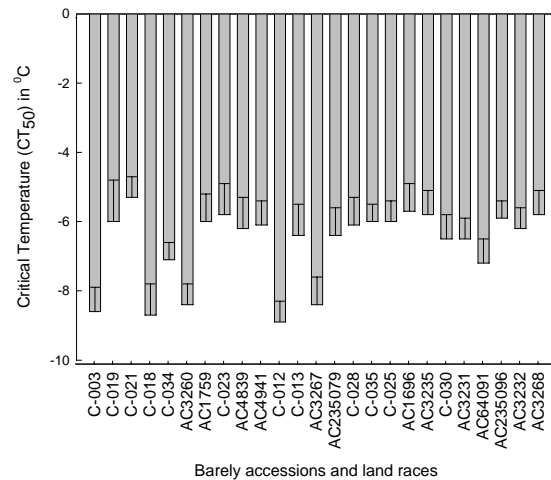


Fig. 1. Critical temperatures ( $CT_{50}$ ) of barley landraces in °C.

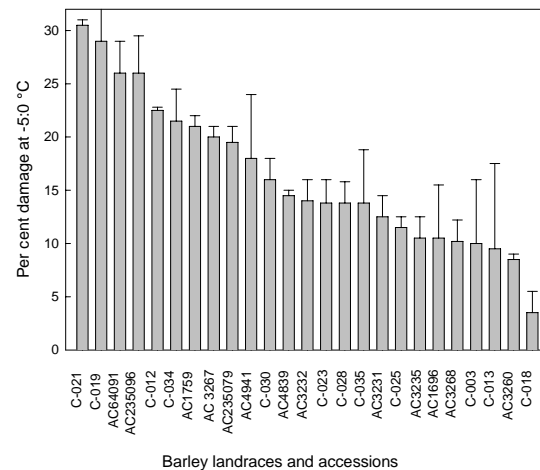


Fig. 2. Percent damage of barley landraces subjected to freezing at -5°C.

### Altitude of provenance and frost hardiness of the races

Figure 3 shows the frost damage vs subzero temperature relations of a representative selection of eight barely landraces, which had been collected from different altitudes, in order to examine the hypothesis that the degree of frost hardiness correlates positively with the altitude at which the landrace is grown. The curves show that relatively frost hardy landraces were collected from both high and low altitudes.

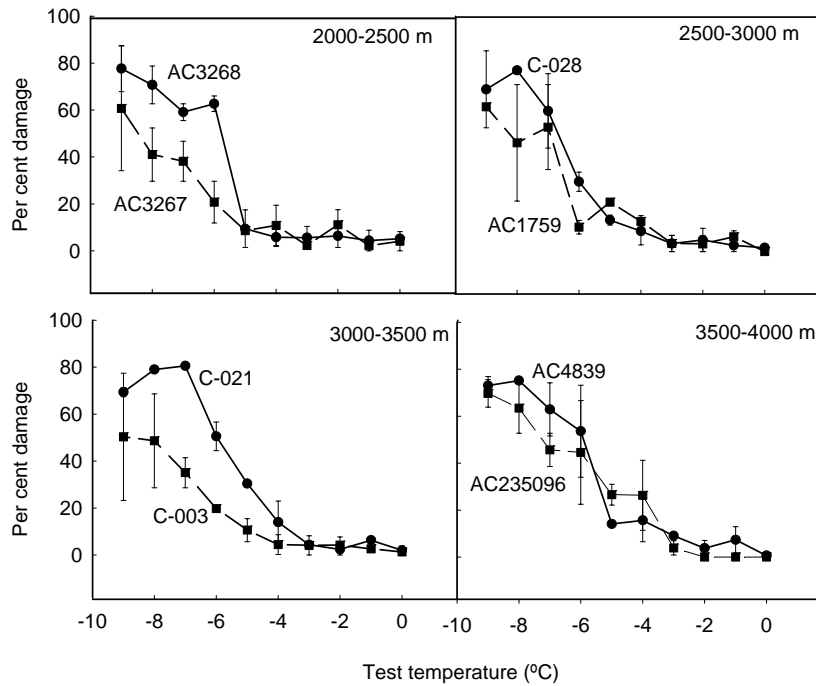


Fig. 3. Comparison of percent damage of frost sensitive and tolerant types of barley landraces collected from similar altitudes.

Therefore a direct context of cold hardiness and altitude of growth could not be demonstrated. A similar result was obtained when the altitude of seed collection was related with the percent damage of all samples after exposure to a minimum temperature of  $-5^{\circ}\text{C}$  in the freeze-thaw cycle (Fig. 4). Data presented in both figures (Figs 3 and 4) indicate that altitude of collection did not influence tolerance of the landraces to frost.

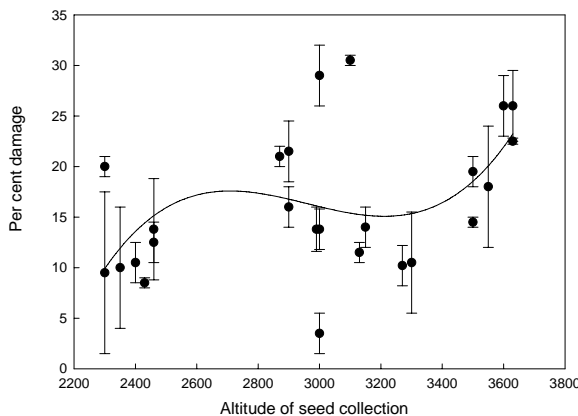


Fig. 4. Relations between percent damage at  $-5^{\circ}\text{C}$  and altitude of collection of barley landraces.

*Cold-acclimation and the potential of recovery from freezing: Photosynthetic quantum efficiency in the course of a freeze-thaw cycle*

Samples from plants grown for 6 weeks in the tropical-alpine greenhouse or plants that had been transferred for 10 days into the phytotrone at a constant temperature of  $2^{\circ}\text{C}$  were used for investigating the potential for cold acclimation. In the same experiment recovery from a freezing event could be determined. Plants grown in the normal greenhouse served as controls. Cold-acclimation was assessed with six landraces which were selected based on their resistance (C-003, C-018, AC3260) or susceptibility (C-012, C-019, AC3267) to a temperature of  $-5^{\circ}\text{C}$  as determined by membrane leakage. Chlorophyll *a* fluorescence was measured and the effective quantum yield of PSII was used as parameter. Chlorophyll *a* fluorescence was measured at 15 temperatures in the course of a freeze-thaw cycle with a special chamber (see Materials and Methods). Figure 5 shows the effective quantum yield and its change in the freeze-thaw cycle of plants of the six landraces after the different frost hardening pre-treatments.

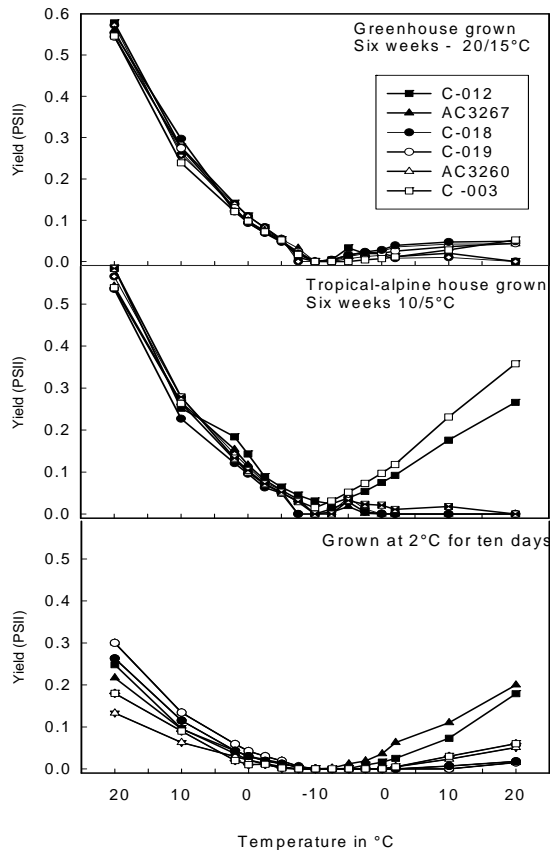


Fig. 5. Quantum yield of PSII of leaf segments of six barley landraces taken at various temperatures in a freeze-thaw cycle starting at 20°C, followed by step wise cooling and slowly re-warming back to 20°C. The landraces were grown in the greenhouse (20/15°C), in the tropical alpine house (12/0°C) and in a phytone for ten days (2°C).

The effective photosynthetic quantum yield of the control plants from the normal greenhouse (measured at the growth temperature of 20°C) was around 0.55. The yield approached zero when the temperature was decreased and there was no recovery of effective quantum yield when the temperature was raised again. When grown in the tropical alpine greenhouse, plants of all six landraces showed the same effective quantum yield and the same rate of decline of quantum yield with temperature as the controls, and at -10°C the yield was again close to zero. While the effective quantum yield of four of the landraces remained low during the re-warming phase (C-018, C-019, AC3260, AC3267) two of the samples (C-003 and C-012) recovered immediately to about 60% of their initial values. When plants of the six landraces were acclimatized in the phytotrone for 10 days at 2°C, significant differences were observed in the generally lower initial yield (0.13

to 0.3), in response to cooling and subsequent recovery. All samples showed recovery during the warming period, although to different extents. Two of the landraces (C-012 and AC3267) recovered to almost 100% of the (relatively low) initial photosynthetic yield. Two others (C-003 and AC3260) recovered by about 50% to the initially lower yield. C-018 and C-019, which had the highest initial effective yields, recovered least, reaching less than 20% during the re-warming period. Thus, landrace C-012 had the best recovery irrespective of having been pre-hardened in the tropical alpine greenhouse or in the phytotrone at 2°C. Landrace C-003 showed good recovery when grown in the tropical-alpine greenhouse, but performed less effectively when pre-treated at 2°C. In contrast, another landrace, AC3267, which did not recover from freezing when grown in the tropical alpine greenhouse, recovered well when the plants were maintained at 2°C prior to the freeze-thaw cycle.

#### Date of ear emergency

The date of ear emergence of selected landraces used in the experiments was followed by growing them in the greenhouse (20/15°C) in order to relate maturity to climate situations of the area of collection. Figure 6 shows that the landraces divided into two groups. The first group had ear emergency in 45 to 50 days, whereas the other group took 70 to 95 days. Two of the cold resistant landraces, C-003 and C-018, had early ear emergence while the other C-012 had late ear emergence indicating late maturation.

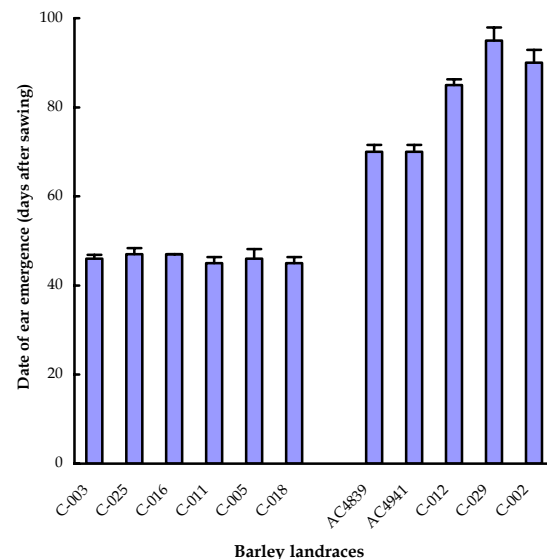


Fig. 6. Time after sowing selected barley land races when ear emergence was observed. The plants were grown in a green house at 20/15 °C day/night.

## DISCUSSION

Ethiopian upland barely landraces investigated in the present study showed considerable differences in their response to subfreezing temperatures and in their acclimation potential to cold. The critical temperatures for survival for most of the 25 samples ranged between -5.5 and -8.8°C. Because there are no earlier reports on frost tolerance of tropical barley races, the present values may be compared to published values for barley from temperate regions. The values are lower than those reported by Mahfoozi *et al.* (2004) for spring barley (between -3 and -6°C) and are more comparable to values for winter barley (between -5 and -14°C, Levitt, 1980). Interestingly, the critical temperature for most of the tolerant landraces (C-003, C-012, C-018 and AC3260, AC3267) was very similar to that of *Chenopodium quinoa* (-8°C), the major staple crop of the tropical Andes which is grown at comparable altitudes (Jacobsen *et al.*, 2005). Sensitivity of the Ethiopian barley landraces to subfreezing temperatures differentiated best at a test temperature of -5°C. A similar observation has been made with other crop cultivars in the Andes, the critical temperatures of which are between -5 and -6°C (Bois *et al.*, 2006). We suggest that -5°C may be used as a general test temperature for selecting tropical highland crop cultivars for frost tolerance. All of the investigated barley landraces when grown at temperatures above +15°C survived that frost temperature, although with differing extent of damage that was in the range of 3 to 30%.

In the present study, no relation could be found between the altitude from where the seeds had been collected and the degree of frost tolerance of the plants. On a first glance, it is surprising that the farmers obviously do not select for frost tolerance of their landraces. One reason may be that extreme frost events, which could destroy the entire harvest, are apparently rare, and that farmers prefer to manage frost risks by trusting in a diversity of landraces rather than in a single locally adapted cultivar (Bois *et al.*, 2006). Another reason may be found in the capability of frost hardening of several of the investigated landraces from high as well as from middle altitudes (2300–3630 masl).

Other factors, which may be likewise important for the selection of races, are the productivity and the maturation time of the crop. Barley landraces investigated in the present study were found to belong to both the early maturing and late maturing groups and more and less frost tolerant varieties were found in both groups. Nocturnal frost in Ethiopian highlands is regular in September/October and December/January (Asmare Yallew *et al.*, 1998). While the early maturing landraces will face frost (between 12 p.m. and 6 a.m.) only during maturation in September and October, late maturing types may have frost both in September/October and December/January. The two most common types of frost are radiative and convective, also known as white and black frost, respectively. White frost, which occurs in September/October, is less severe as it occurs after the long rains at high relative humidity and relatively high dew point temperature (Ruiz, 1995; Jacobsen, 2005). With this type of frost, water vapour condenses and freezes on the leaf surface. Freezing of water on the plant surfaces usually triggers also crystallization inside the tissue, but the rigidity of the cell walls substantially reduces intercellular ice formation and thus freezes desiccation of the barley mesophyll cells (Hansen and Beck, 1988). Black frost occurs when the air is dry, typical of December/January in northern Ethiopia, with temperatures not reaching the low dew point temperature. In this case, dewfall is rare and the air temperature drops rapidly, which could trigger lethal intracellular ice formation (Hansen and Beck, 1988). Thus, frost during this period may have a severe effect on the crop.

Low-temperature acclimation is an ability of plants to adjust their physiology when exposed to temperatures below a specific threshold. This is the most common mechanism that plants have evolved for adapting to cold stress. Two types of cold hardening treatment have been employed to assess the capability of barley races to acclimate to freezing: Growth under the typical tropical alpine climate in a special greenhouse, and a long-term exposure to an artificially constant low temperature in a phytotrone. The latter also retards growth of the plants significantly. Due to experimental limitation and in order to get more data that are detailed on the effects of frost,

chlorophyll *a* fluorescence was used for assessment of cold resistance and recovery from freezing instead of the conductivity method. Both pre-treatments of the plants resulted in frost hardening, although not with all samples and not in a similar way. More of the landraces responded better to the permanent low temperature pre-treatment. Only two of the alpine greenhouse grown races showed good recovery of the photosynthetic quantum yield in the freeze-thaw cycle. It should be noted that in these experiments the plants were cooled to  $-10^{\circ}\text{C}$ , a temperature that causes lethal damage of all non-acclimatized plants, as is evidenced by the controls. Interestingly, the plants that had been grown under the tropical alpine climate showed the same high photosynthetic quantum yield under daytime conditions as the non-acclimatized controls, while those hardened at  $2^{\circ}\text{C}$  had significantly lower quantum efficiency. All samples, frost hardened or not, showed a strong drop of the photosynthetic quantum yield with decreasing temperatures but with still some efficiency even below  $0^{\circ}\text{C}$ . When frozen, photosynthetic electron transport ceases (see also Bodner and Beck, 1987). In winter barley when leaves are subjected to  $-10^{\circ}\text{C}$ , 80% of the mesophyll water is deposited as ice in the intercellular spaces (Hansen and Beck, 1988). Assuming similar relations in the plants investigated in this study inhibition of photosynthesis at this temperature due to freeze desiccation is not surprising. The immediate start of photosynthesis upon re-warming in the frost hardened samples indicates that no damage has been inferred by freezing of the leaf tissue. Maximal frost hardening was observed with those landraces, which also showed the highest degree of cold tolerance in the ion leakage test, with one exception: C-018. This landrace had a  $\text{CT}_{50}$  of  $-8.8^{\circ}\text{C}$  but obviously could not be hardened by the treatments employed in this study. It belongs to the early maturing type and has been collected from a barley field in 3000 m altitude, where mild frost may occur especially during the dry season. Furthermore, it showed extremely small damage at  $-5^{\circ}\text{C}$ , thus differentiating well from all other investigated landraces.

Barley is a cereal which can be grown under several types of stress resulting in physiological water shortage: drought, high salinity and frost

(McKersie and Leshem, 1994; Schulze *et al.*, 2005). Its potential for acclimatizing to that kind of strain is high. Since drought is common in the alpine zones of Ethiopia during the dry season, and due to the cross protection against drought and freezing stress (Close and Bray, 1993; Close, 1997) the level of basic cold tolerance found with all investigated barley landraces is understandable. However, the physiological changes by which cold hardening of several of the investigated races is accomplished will require further studies.

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