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# Feeling the cold: atmospheric CO<sub>2</sub> enrichment and the frost sensitivity of terrestrial plant foliage

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## Abstract

Quantitative palaeoclimate reconstructions from plant fossils using the nearest living relative (NLR) approach axiomatically assume that the climatic limits of plant distributions have largely remained unchanged over at least the past 250 Myr. However, throughout much of the Mesozoic the atmospheric CO<sub>2</sub> concentration is predicted to have been several times greater than the present day, and long-term ( $\geq 5$  yr) experimental CO<sub>2</sub> enrichment studies indicate this has the capacity to increase the frost sensitivity of plant foliage by raising the temperature at which leaf tissues freeze. Furthermore, we have been able to document increases in the leaf freezing temperatures of some woody shrub species in response to the rise in atmospheric CO<sub>2</sub> concentration over the past two centuries, suggesting leaf freezing temperatures are sensitive to below ambient CO<sub>2</sub> concentrations. These data imply that a previously unrealised increase in the frost sensitivity of terrestrial plants may have already occurred owing to anthropogenic impacts on the contemporary global carbon cycle. Possible consequences of these findings for palaeotemperatures generated from the application of the NLR technique to a time-series of floras are discussed, with particular attention given to differential effects resulting from the nature of the leaf freezing–CO<sub>2</sub> relationship. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Palaeobotanists typically use the nearest living relative (NLR) approach as a method for extracting palaeoclimatic signals from plant fossils (Chaloner and Creber, 1990; Jordon, 1997). The NLR approach tacitly assumes that the climatic

limits on plant distributions operating under the present-day climate and atmospheric CO<sub>2</sub> concentration are similar to those operating in the distant past. Determination of some climatic feature thought to limit the distribution of selected plant taxa at the global and regional scale, e.g. low temperatures for gingers and palms, then allows palaeoclimate reconstructions to be made directly from plant fossils of similar, closely related, lineages (Jordon, 1997). In this way, given localities spanning a sufficiently large latitudinal gradient, quantitative estimates of regional palaeoclimates can be obtained to allow inferences about the operation of the global climate system at a specific

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time in the distant past, when the boundary conditions (e.g. continental configuration, solar luminosity, oceanic circulation) were different from now. These reconstructions can then be compared with global-scale palaeoclimate simulations of the same interval by general circulation models as one means of testing the models (e.g. Wing and Greenwood, 1993; Greenwood and Wing, 1995).

The validity of the assumptions underpinning the NLR approach, as applied to vascular land plants, needs examining in relation to atmospheric evolution. Long-term carbon cycle models (Bernier, 1994, 1997, 1998) and palaeo-CO<sub>2</sub> proxies (McElwain and Chaloner, 1995; Beerling and Woodward, 1997; Ekart et al., 1999) indicate that the mass of carbon in the atmospheric reservoir during the Mesozoic and Cenozoic was probably several times greater than the present-day. In general, geochemical models predict a high (1500 ppm) but declining pattern of CO<sub>2</sub> change between 200 and 50 million years ago, with estimates derived from isotopic analyses of fossil soils suggesting a decline in CO<sub>2</sub> concentrations from 3000 to 1000 ppm over this time (Ekart et al., 1999). It follows therefore that Mesozoic and Cenozoic plant fossils provide a record of plant growth characterised by a CO<sub>2</sub>-rich atmosphere. Given that a substantial database of experimental evidence has demonstrated that woody plant growth in elevated CO<sub>2</sub> (see Drake et al., 1997; Norby et al., 1999 for reviews) considerably alters a wide range of plant physiological processes, similar effects will be expected to feed through and alter the climatic determinants of land plant distribution in the geological past.

An important, but largely neglected, component of this current CO<sub>2</sub> research effort is the effect of elevated CO<sub>2</sub> concentrations on plant low temperature/freezing tolerance, a feature critical to determining their performance and distribution (Long and Woodward, 1988). Investigation of the nature of high CO<sub>2</sub>-low temperature interactions in plants is required to understand its possible influence on quantitative palaeotemperature estimates from plant fossils using the NLR approach (Beerling, 1998). Here we take up this theme by first reviewing recent experimental data that specifically investigates the impact of elevated

CO<sub>2</sub> on the frost sensitivity of terrestrial plant foliage. The potential for the increase in atmospheric CO<sub>2</sub> over the past two centuries (Friedli et al., 1986; Keeling and Whorf, 1994) to influence the frost sensitivity of vegetation is then examined through the development of a new approach for documenting shifts in leaf freezing temperatures from historical archives of leaves. Finally, we discuss the consequences of these high CO<sub>2</sub>-low temperature interactions for palaeoclimate curves generated over intervals of geological time using the NLR approach at times of fluctuating atmospheric CO<sub>2</sub> concentrations.

## 2. Experimental evidence for CO<sub>2</sub> effects on plant frost sensitivity

The capacity of atmospheric CO<sub>2</sub> to alter the temperature at which leaves freeze was first reported from a CO<sub>2</sub> enrichment experiment with replicated open-top chambers using seedlings of the frost-hardy evergreen *Eucalyptus pauciflora* (Lutze et al., 1998). The occurrence of a natural frosting event (to  $-5^{\circ}\text{C}$ ) during the spring, following over-wintering of seedlings, resulted in a significantly ( $P < 0.001$ ) greater number of plants showing visible leaf damage with CO<sub>2</sub> enrichment (68% at 700 ppm CO<sub>2</sub>) compared to the ambient grown counterparts (37% at 350 ppm CO<sub>2</sub>). Measurements of ice nucleation temperatures [i.e. temperature at which intracellular ice crystal formation occurs, *sensu*, Sakai and Larcher (1987)] of leaves from both sets of plants revealed that those growing with CO<sub>2</sub> enrichment had ice nucleation temperatures 1.4°C higher than those plants with leaves developing at ambient CO<sub>2</sub>. In this way, the authors were able to establish the important link between leaf damage and CO<sub>2</sub> effects on ice nucleation temperatures.

The largely unknown extent to which this phenomenon operates in other species has more recently been addressed by new measurements made on a range of dwarf ericaceous taxa grown with and without CO<sub>2</sub> enrichment in the sub-Arctic over the past 7 yr (Beerling et al., 2001). These measurements revealed that three of the four taxa investigated had significant ( $P < 0.001$ ) season-

long increases of several degrees centigrade in the ice nucleation temperatures of leaves of those plants growing in elevated CO<sub>2</sub> (650 ppm) compared with those from ambient CO<sub>2</sub> (350 ppm). A further dataset provides information on the effects of long-term growth in elevated CO<sub>2</sub> on saplings of the deciduous 'living fossil' taxon *Ginkgo biloba* (Terry et al., 2000). *Ginkgo* is particularly interesting in this respect since it represents one of the most ancient seed plants and had a circumpolar distribution in the Northern and Southern hemispheres between the late Mesozoic and early Tertiary (Rothwell and Holt, 1997). From a palaeobiological perspective therefore *Ginkgo* is a taxon with a probable long history of exposure to elevated CO<sub>2</sub>. As with the sub-Arctic woody shrubs, *G. biloba* showed significantly ( $P < 0.01$ ) higher ice nucleation temperatures after 5 yr growth in a CO<sub>2</sub>-enriched environment compared to those from ambient CO<sub>2</sub>. Moreover, this effect persisted between mid-summer (June) and late in the growing season when leaves had begun to senesce (October).

Tests of leaf survival on the Arctic shrubs (Beerling et al., 2001) and *Ginkgo biloba* (Terry et al., 2000) showed that leaves of all species were killed after cooling to temperatures below their ice nucleation thresholds, an effect with different implications for plant performance in the field, depending on the timing of a severe frosting event. Plants in elevated CO<sub>2</sub> subjected to a late spring frost will have photosynthesis abruptly terminated and quite likely lose a greater proportion of their leaf surface area than those operating under an ambient CO<sub>2</sub> atmosphere, with the consequence that photosynthetic carbon fixation will be curtailed. In contrast, an early-autumn frost would result in leaf death and abscission causing the loss of stored non-structural assimilates and ca. 50% of leaf mineral nutrients that are usually recovered during normal senescence (Körner and Larcher, 1988). These limitations on plant performance will operate in addition to increased photo-inhibition following non-lethal natural freezing events resulting from growth in elevated CO<sub>2</sub> (Roden et al., 1999; Terry et al., 2000).

The results from these long-term CO<sub>2</sub> enrichment studies extend the findings of Lutze et al.

(1998) in several key ways. They show that (i) the response can be observed in mature vegetation grown with CO<sub>2</sub> enrichment, since the sub-Arctic CO<sub>2</sub> enrichment experiments were performed on natural undisturbed stands of vegetation, (ii) it is not apparently confined to plants with either an evergreen or deciduous habit and (iii) it is not a transient feature of plant growth in a high CO<sub>2</sub> environment limited to the early or late part of the growing season. Collectively these points suggest a generality in the effects of CO<sub>2</sub> on foliage frost sensitivity not previously realised, and so point to the strong potential for vegetation growing in the high CO<sub>2</sub> atmosphere of the Mesozoic to have been more frost sensitive than vegetation experiencing today's climate and CO<sub>2</sub>. One potential net consequence of this phenomenon might be that the cumulative effects of exposure to repeated frosting events in a high CO<sub>2</sub> atmosphere were sufficient to alter the cover, biomass production and distribution of plants in the Mesozoic such that their climatic limits at that time were different from those seen in taxa of modern vegetation communities.

### 3. Effects of historical and altitudinal CO<sub>2</sub> changes on foliage frost sensitivity

The above summary of experimental data raises the question as to whether plant frost sensitivity has been measurably altered by the increase in atmospheric CO<sub>2</sub> over the past two centuries (Friedli et al., 1986; Keeling and Whorf, 1994). Herbarium collections provide one source of material for investigating this issue, assuming the recovery of representative ice nucleation temperatures is possible after re-hydration of dried leaves. We first tested for this possibility in a pilot study by collecting and drying (for 6 weeks) *Ginkgo biloba* leaves from ambient and elevated CO<sub>2</sub> environments, with the assumption that this procedure approximately reproduced the processes involved in leaf collection and storage in herbaria. These leaves were then re-hydrated in de-ionised water for 48 h and the ice nucleation temperatures determined from exotherm characteristics as described previously (Beerling et al., 2001; Terry et

al., 2000). In all cases, ice nucleation temperatures measured on this material were within  $\pm 0.5^\circ\text{C}$  of those obtained on fresh leaves of the same species at the same time of collection.

From these results, it was assumed that representative leaf ice nucleation temperatures could be recovered from re-hydrated leaves and a preliminary study made on historical leaf sequences of four species of dwarf woody shrubs from Scotland (*Salix herbacea*, *Myrica gale*, *Salix repens*, and *Betula nana*) available for analysis from the University of Sheffield herbarium dating from 1768 to 1968 AD. This period covered a 45-ppm rise in atmospheric  $\text{CO}_2$  (from 277 to 322 ppm) allowing us to test for any historical changes in leaf freezing temperatures of these species. Leaf ice nucleation temperatures were measured after re-hydration of the herbarium specimens for 48 h, as described above. The resulting correlation between ice nucleation temperatures and the atmospheric  $\text{CO}_2$  concentration at the time of growth, as determined from ice cores (Friedli et al., 1986) and instrumental records (Keeling and Whorf, 1994) is depicted in Fig. 1. Three of the four species showed significant positive relationships be-

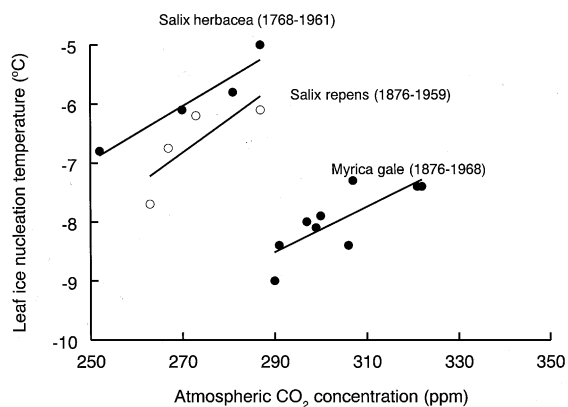


Fig. 1. Relationship between leaf ice nucleation temperatures measured on re-hydrated herbarium leaves of three shrub species and the estimated concentration of atmospheric  $\text{CO}_2$  during the year of growth. Values are means of measurements made on two to three leaves per sampling date. Regression details: *Salix herbacea*, slope = 0.046, intercept =  $-18.55$ ,  $r = 0.96$ ; *S. repens*, slope = 0.057, intercept =  $-22.10$ ,  $r = 0.81$ ; *Myrica gale*, slope = 0.039, intercept =  $-19.69$ ,  $r = 0.79$ .

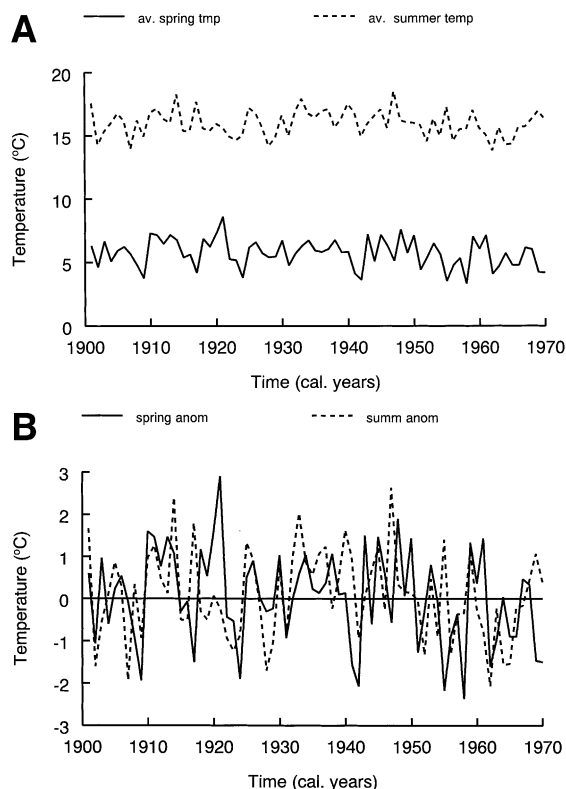


Fig. 2. Past changes in (A) absolute spring (March–April–May mean) and summer (June–July–August mean) land surface temperatures over northern England ( $53^\circ\text{N}$ ) and (B) expressed as anomalies relative to the mean for each time series. Data from New et al. (2000).

tween atmospheric  $\text{CO}_2$  and ice nucleation temperatures of leaves examined in this way (Fig. 1) and the direction of this response is consistent with those data obtained from experiments on woody shrubs grown in elevated  $\text{CO}_2$  (Fig. 1) (i.e. a rise in leaf ice nucleation temperature with an increase in  $\text{CO}_2$  concentration). *Betula nana*, the fourth species investigated from herbarium materials, showed no marked shifts in nucleating temperatures with values of  $-6.0^\circ\text{C}$  in 1911 AD and  $-6.6^\circ\text{C}$  in 1936 AD.

It is well established that leaf freezing temperatures can be influenced by growing season climate due to its impacts on physiological cold hardening processes (Sakai and Larcher, 1987; Larcher, 1995). It is possible therefore that the

historical increases in leaf freezing temperatures (Fig. 1) may have been driven by inter-annual climate variability rather than the rise in CO<sub>2</sub> over this time. We tested this suggestion by calculating spring (March–April–May) and summer (June–July–August) temperature trends from climate data for the 20th century over northern England (New et al., 2000), close to the geographical source of the herbarium specimens. The resulting time-series showed no obvious trends when expressed either as absolute values (Fig. 2A) or as anomalies relative to the long-term mean (Fig. 2B). Neither dataset shows any strong persistent anthropogenic warming signal because this has occurred post-1980 (Jones et al., 1999). For those three species showing an increase in leaf freezing temperatures with CO<sub>2</sub>, no significant correlations emerged with either spring (Fig. 3A) or summer (Fig. 3B) temperatures, suggesting the observed inter-annual climate variability is unlikely to explain the relationship between historical CO<sub>2</sub> change and leaf freezing temperatures (Fig. 1).

There is however also the possibility that undetermined age-related degradation effects on herbarium leaf properties in some way influenced the measured ice nucleation temperatures. In an effort to test for this effect, natural CO<sub>2</sub> gradients that occur across mountains were utilised by analysing herbarium leaves collected in the same year but across a wide range of different altitudes. Leaves from four species with suitable collections in the University of Sheffield herbarium were obtained: *Salix herbacea* (100–1000 m asl), *Lychnis alpina* (30–800 m asl), *Vaccinium uliginosum* (20–800 m asl) and *Betula nana* (40–1200 m asl). A wide altitudinal range for each species was achieved with leaves from collections at different localities across N.W. Europe. Responses of plants to altitudinal changes in CO<sub>2</sub> are not strictly comparable to those obtained from changes in the atmospheric CO<sub>2</sub> concentration since mole fraction is constant with altitude but partial pressure (and temperature) decreases (Gale, 1972). Nevertheless, the approach provides one means of controlling for the effects of sample age on measured ice nucleation temperatures.

Leaf ice nucleation temperatures were measured after re-hydration of the herbarium leaves for

48 h, as with the historical sequences. The partial pressure of atmospheric CO<sub>2</sub> under which the leaves developed was calculated from the global mean CO<sub>2</sub> concentration ([CO<sub>2</sub>] in ppm), at the time sample collection, and correcting it for altitude by calculating the atmospheric pressure ( $P_z$  in Pa) at the altitude ( $z$  in m) recorded on the herbarium sheet (Jones, 1992):

$$P_z = 101325 \times \exp\left(\frac{-M_a \times g \times z}{\mathcal{R} \times t}\right) \quad (1)$$

where  $M_a$  is the molecular weight of air ( $28.964 \times 10^{-3} \text{ kg mol}^{-1}$ ),  $g$  is the acceleration due to gravity ( $9.8 \text{ m s}^{-2}$ ),  $t$  is the temperature (°C) and  $\mathcal{R}$  is the gas constant ( $8.3144 \text{ J mol}^{-1}$

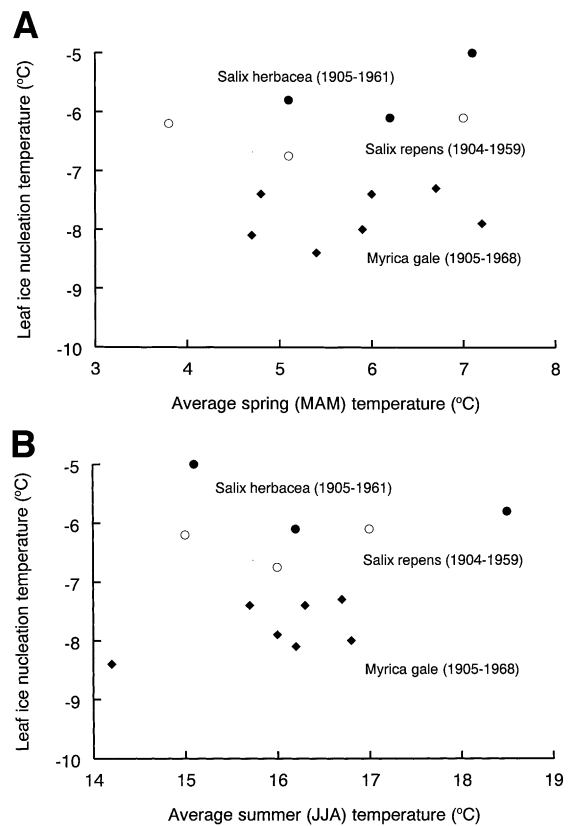


Fig. 3. Relationship between measured ice nucleation temperatures of herbarium leaves for different species of dwarf shrubs shown in Fig. 1 and (A) spring (MAM) and (B) summer (JJA) temperatures of the specific year in which they were collected. None of the correlations were significant. Species codes: filled circles: *Salix herbacea*, open circles: *S. repens*, filled diamonds: *Myrica gale*.

$\text{K}^{-1}$ ). The  $\text{CO}_2$  partial pressure ( $p(\text{CO}_2)$  in Pa) at a given atmospheric pressure is then calculated from:

$$p(\text{CO}_2) = \frac{P_z}{101325} \times [\text{CO}_2] \quad (2)$$

The ice nucleation temperatures of leaves from different altitudes show a strong linear increase when plotted against the calculated partial pressure of  $\text{CO}_2$  at the time of growth (Fig. 4). The rise in leaf freezing temperatures with  $p(\text{CO}_2)$  would therefore correspond to increased frost sensitivity of shrub foliage at lower altitudes. But, we recognise that there may also be a correlated effect of altitude on temperature, with cooler temperatures prevailing at higher altitudes (Barry, 1992) leading to lower ice nucleation temperatures. Therefore, we tested for an altitude-related climatic effect in a limited way by measuring the freezing temperatures on basal rosette leaves and leaves of the flowering shoots of *Lychnis alpina*, with the latter developing under a warmer (summer) climatic regime. It emerges that both sets of *L. alpina* leaves showed the same response (Fig. 4), providing some further support that  $\text{CO}_2$  impacts apparently override the effects of growing season climate (cf. Fig. 3). It is notable that *Salix herbacea* showed the same direction of response to a rise in  $p(\text{CO}_2)$  observed from historical sequences of herbarium leaves (Fig. 1) and the response of *Vaccinium uliginosum* was consistent with the data from plants grown in situ with  $\text{CO}_2$  enrichment in the sub-Arctic (Beerling et al., 2001). This suggests that a  $\text{CO}_2$ -related impact on leaf freezing temperatures for these species at least can be brought about by changes in the concentration and partial pressure of the gas. *Betula nana* showed no changes in ice nucleating temperature across the altitudinal range 100 m ( $-7.6^\circ\text{C}$ ) to 1200 m ( $-7.8^\circ\text{C}$ ) and this is also consistent with the earlier finding that showed no measurable historical changes.

The altitudinal  $p(\text{CO}_2)$ –leaf freezing relationship (Fig. 4) provides one possible explanation for the differential survival of lettuce seedlings artificially planted across wide altitudinal gradients on different mountains in the Southern hemisphere (Halloy and González, 1993). Those

authors reported increased survival of seedlings at higher altitudes, where  $p(\text{CO}_2)$  must have been lower, due to the drop in atmospheric pressure with the monotonic fall in gravitational attraction towards Earth's surface. Indeed, replotting the data of Halloy and González (1993) against atmospheric  $\text{CO}_2$  partial pressure, calculated from Eqs. 1 and 2, shows altitudinal trends in survival consistent with the notion that leaf freezing temperatures declined with  $p(\text{CO}_2)$  (Fig. 5).

Continued future increases in the concentration of atmospheric  $\text{CO}_2$  are expected (IPCC, 1996) and have already occurred ahead of a marked climatic warming, possibly because of the attenuating influence of the world's oceans (Levitus et al., 2000). Since elevated  $\text{CO}_2$  has been shown experimentally to have rather conservative effects on the timing of bud-burst in temperate tree species (Murray and Ceulemans, 1998) it is of interest to consider those geographical areas where contemporary vegetation periodically experiences ground frosts. A global map of low temperature and frost occurrence on Earth provides this overview (Fig. 6) and shows that regions experiencing episodic frosts down to  $-10^\circ\text{C}$  (stippled areas in Fig. 6) are extensive throughout Australia, South Africa, southern parts of the USA, South Amer-

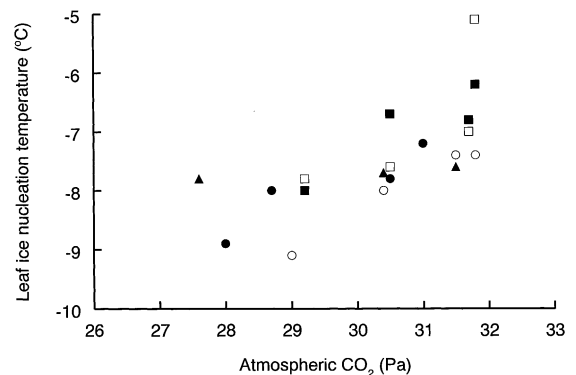


Fig. 4. Correlation between measured ice nucleation temperatures of herbarium leaves collected in the same year (for any given species) and the partial pressure of atmospheric  $\text{CO}_2$  under which they developed at a specified altitude, calculated using Eqs. 1 and 2. Species codes: filled circles: *Salix herbacea*, open circles: *Vaccinium uliginosum*, open and filled boxes: basal rosette and stem leaves of *Lychnis alpina* respectively, filled diamonds: *Betula nana*.

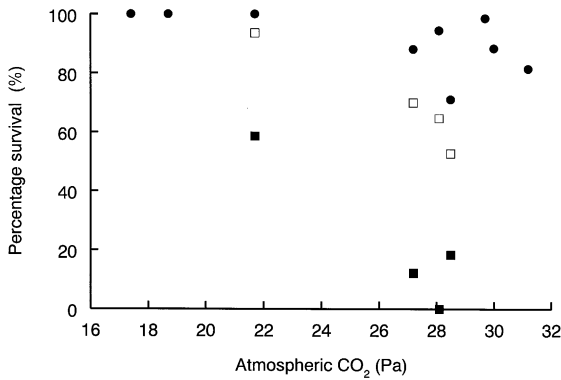


Fig. 5. Age-dependent survival of lettuce seedlings planted across an altitudinal range (1200–6060 m asl) on various Southern Hemisphere mountains in relation to the estimated partial pressure of atmospheric CO<sub>2</sub> under which they developed. Filled circles: survival after 30 days, open boxes: survival after 60 days, filled boxes: survival after 140 days. Calculated from Halloy and González (1993).

riding impression from this map is that a high proportion of plants distributed in the temperate regions of the Northern and Southern hemispheres may be at a greater risk of damage from severe frosting events as the atmospheric CO<sub>2</sub> concentration continues to increase. Obviously not all species will be affected to the same extent since those in the high latitudes typically possess mechanisms for super-cooling tissues, freeze tolerance and ecological strategies to deal with extreme low temperature events (Larcher, 1995). If cultivated plants, particularly crops, show increased frost sensitivity to CO<sub>2</sub> enrichment then, since these are frequently planted up to the extreme limits of their survival, some impacts on agricultural production might ensue.

ica and Asia and parts of Africa. Temperate regions with average annual minimum temperatures between  $-10$  and  $-40^{\circ}\text{C}$  are less extensive and dominate large areas of the Northern Hemisphere land masses (hatched areas in Fig. 6). The over-

#### 4. Implications for palaeoclimate estimates using the NLR approach applied to terrestrial plant fossils

We next consider the potential for a CO<sub>2</sub>-driven change in the sensitivity of leaf freezing temperatures on a geological time-scale. An initial

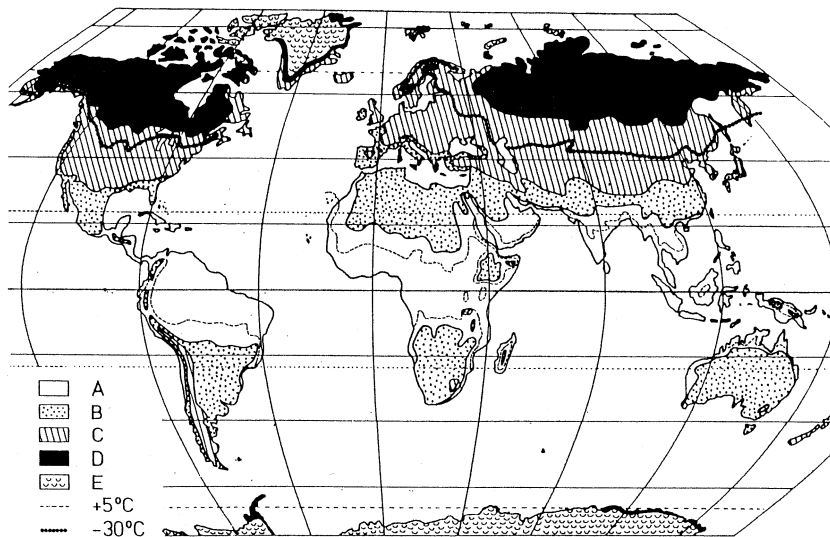


Fig. 6. Global map of low temperature and frost occurrence for the present day climate. Codes: A, frost-free zone; B, episodic frosts down to  $-10^{\circ}\text{C}$ ; C, regions with an average annual minimum temperatures between  $-10$  and  $-40^{\circ}\text{C}$ ; D, regions with average annual minimum temperature below  $-40^{\circ}\text{C}$ ; and E, polar ice and permafrost. Also shown are the  $-30^{\circ}\text{C}$  and  $+5^{\circ}\text{C}$  minimum isotherms. From Larcher (1995).

assessment of this possibility has been made by focussing on the response of the woody shrub *Vaccinium uliginosum* because this is the only species for which it is possible to construct a CO<sub>2</sub>–leaf freezing response curve across the range 300–600 ppm. It is, however, not clear whether the resulting response (Fig. 7A) is linear or curvilinear, so lines have been fitted to account for both possibilities. The aim of the current exercise, to determine the possible effects of CO<sub>2</sub> variations on trends in leaf freezing temperatures over the past 250 Myr, has then been achieved by forcing these two response curves (Fig. 7A) with predicted long-term trends in atmospheric CO<sub>2</sub> change over this time (Fig. 7B).

From these calculations it can be seen that quite large differences ( $\sim 4^{\circ}\text{C}$ ) in leaf freezing temperatures could have existed (relative to the modern value), due solely to changes in atmospheric CO<sub>2</sub> concentration over the past 250 Myr, with vegetation in general being more sensitive to frosts in the past compared to now (Fig. 7C). A higher-than-present concentration of atmospheric CO<sub>2</sub> prevailing over the past 250 Myr therefore could have favoured the evolution of plants with a reduced cold tolerance compared to now. This evolutionary development would mean that estimating minimum temperatures from the current climatic range of frost-sensitive taxa such as palms and gingers (e.g. Greenwood and Wing, 1995) will yield values that are too

cool. The curves (Fig. 7C) further show that provided the atmospheric CO<sub>2</sub> concentration remained above some threshold value (here 600 ppm), a CO<sub>2</sub> bias on past temperature estimates would be constant. However, if the atmospheric CO<sub>2</sub> concentration dropped below this threshold, a variable bias would be introduced (Fig. 7C).

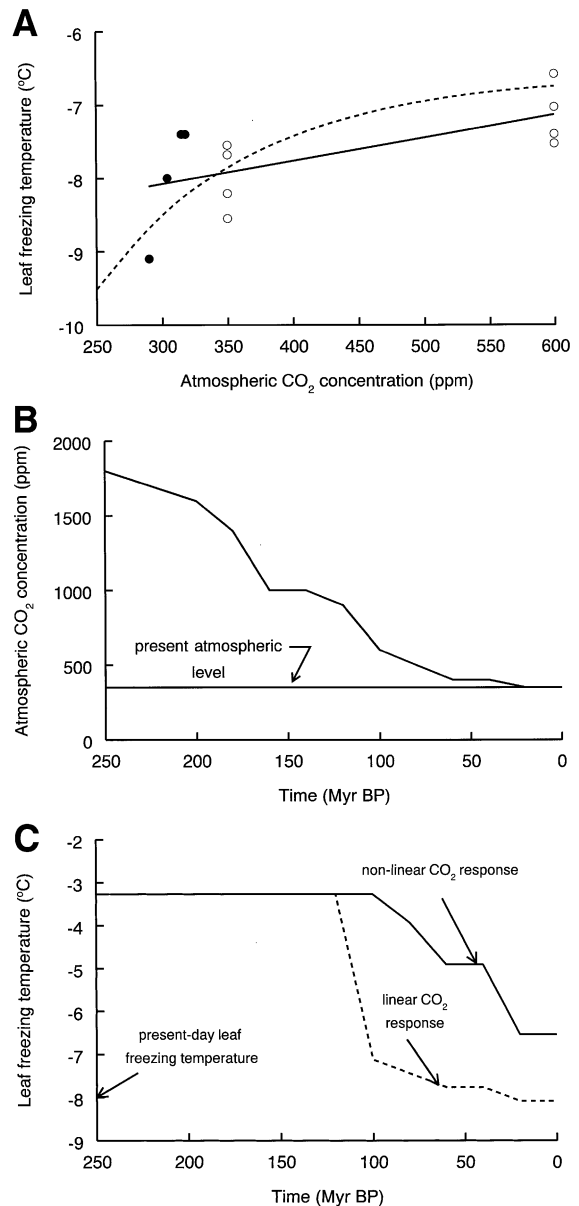


Fig. 7. (A) Response leaf ice nucleation temperatures of the dwarf woody shrub *Vaccinium uliginosum* to atmospheric CO<sub>2</sub>. Filled circles: experimental data (from Beerling et al., 2001), open circles: data from herbarium leaves (Figs. 1 and 4). Note that ice nucleation temperatures of leaves from living *V. uliginosum* grown at 350 ppm CO<sub>2</sub> are close to those of herbarium materials collected from plants growing at low altitude. Regression details: linear line (solid line), slope = 0.003208, intercept = -9.046,  $r = 0.64$ . Non-linear line (dashed line) ice nucleation temperature ( $^{\circ}\text{C}$ ) =  $2.53 \times 10^{-6} \times \text{CO}_2 - (-7.3 \times 10^{-3}) + (-1.97 \times 10^3) \times 1/\text{CO}_2 + 1$ , where CO<sub>2</sub> is the growth CO<sub>2</sub> concentration in ppm which is less than 600 ppm. (B) Generalised long-term trend in atmospheric CO<sub>2</sub> over the past 250 Myr BP (from Berner, 1998). (C) Calculated changes in leaf freezing temperature over the past 250 Myr as predicted by either the linear (solid line) or curvilinear (dashed line) relationships given in (A) and forced with the CO<sub>2</sub> trend in (B).



Such an effect might be important if temperatures were being estimated from floras of different ages during which time the atmospheric CO<sub>2</sub> level had changed, e.g. across the Cenomanian/Turonian boundary (Kuypers et al., 1999).

It is interesting to consider that suggest plant foliage would have greater cold tolerance, at times when the atmospheric CO<sub>2</sub> concentration declined to below present-day values. Several palaeo-CO<sub>2</sub> proxy indicators reconstruct atmospheric CO<sub>2</sub> concentrations for much of the late Miocene (16–5 Ma) of between 200 and 300 ppm (Van de Burgh et al., 1993; Pagani et al., 1999) suggesting a bias of opposite sign (i.e. too warm) in palaeotemperatures using the NLR approach might indeed be a real possibility when applied to this epoch.

It is, of course, a major extrapolation to assume all species of vascular land plants show a pattern of leaf freezing sensitivity in response to CO<sub>2</sub> change which is similar to that of *Vaccinium uliginosum*. For the purposes of this theoretical ‘what if’ analysis, the approach nevertheless illustrates that the direction and magnitude of CO<sub>2</sub>-related bias introduced into palaeoclimatic estimates from plant fossils using the NLR approach might be rather complex. The experimental and herbarium data point to the urgent need for determining changes in foliage sensitivity to atmospheric CO<sub>2</sub> for those frost-sensitive groups of plants typically employed in quantitative NLR analyses, across a range of atmospheric CO<sub>2</sub> concentrations likely to have been encountered during their evolutionary history.

## 5. Conclusions

The phenomenon of increased leaf ice nucleation temperatures in the evergreen eucalypt *Eucalyptus pauciflora*, resulting from growth in elevated CO<sub>2</sub>, has now been found to be quite widespread. A preliminary analysis of herbarium materials (Figs. 1 and 4) indicates that the response extends to CO<sub>2</sub> concentrations below current ambient. Both results, in combination with geochemical model and palaeo-CO<sub>2</sub> data, suggest that CO<sub>2</sub>-selection over millions of years is a real

possibility with the plant cold tolerance being somewhat different from present-day vegetation.

Central to further developing our understanding of this work is a mechanistic explanation for the action of CO<sub>2</sub> on the freezing temperatures of leaf tissues. To increase the ability to super-cool leaf and stem tissues further by avoiding or delaying ice nucleation, requires a change in the compartmentalisation of water into cells and/or cell wall structure, particularly lignin and calcium content (DeHayes et al., 1997). DeHayes et al. (1997) showed that in red spruce needles, the onset of cold hardening was accompanied by an increase in mesophyll cell membrane Ca ion concentration, with Ca postulated to act as a messenger for increasing unsaturated fatty acid incorporation into the membrane. If Ca ions played this type of role in the CO<sub>2</sub> effects summarised here, then a rise in ice nucleation temperatures with CO<sub>2</sub> enrichment would be accompanied by a decrease in foliar Ca concentrations, and indeed this effect has been reported for sour orange trees (*Citrus aurantium*) grown with CO<sub>2</sub> enrichment for 7 yr (Peñuelas et al., 1997) and from analyses of herbarium leaves of tree, shrub and herb species from N.E. Spain (Peñuelas and Matamala, 1993). So several plausible sites of action for CO<sub>2</sub> exist, and these warrant further investigation.

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