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The effects of temperature on stem elongation of the alpine and prairie ecotypes of *Stellaria longipes*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Effects Of Temperature On The Stem Elongation Of Alpine And Prairie Ecotypes Of *Stellaria longipes*" by Dang Thi Thu Thuy in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Stellaria longipes is a polymorphic herbaceous perennial species that shows a large degree of phenotypic plasticity reflected in morphological changes. Stem elongation provides *S. longipes* genotypes with the fitness to survive in their respective environments. This thesis used *S. longipes* as a model system to study the effects of temperature on stem elongation of alpine and prairie ecotypes. In this study, the morphological, physiological, and histological aspects of stem elongation were examined to illustrate the influential role that temperature plays throughout growth of *S. longipes* and the close interaction between temperature, hormone signalling and cellular structures. Results showed that temperature exerted different effects on the growth of alpine and prairie ecotypes. Variation in the elongation of both ecotypes under the same conditions is most likely due to differences in the ability of each ecotype to respond to specific temperature regime. Differences in the timing of cell wall thickening, deposition of phenolic compounds and cross-linking of cell wall material and especially the dynamic of cortical MTs might cause differences in the elongation, division and stretching ability of the cells, resulting in differences in the stem elongation of alpine and prairie ecotypes of *S. longipes* grown under contrasting temperature regimes. Plant growth regulators, including ethylene, IAA and GA_3, appeared to play a significant role in temperature-induced stem elongation of alpine and prairie ecotypes. Differences in stem elongation of alpine and prairie ecotypes were caused by differences in biosynthesis and sensitivity to ethylene, IAA and GA_3.

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With greatest gratitude and love, I dedicate this thesis to:

My grandparents for their beautiful love and memories

My parents for their unconditional support and encouragement throughout my academic pursuits

and

My fiancé, Đôn, for his continuous love and support.

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Abbreviations

A3	AMO 1618 at concentration of 10^{-3}M
A4	AMO 1618 at concentration of 10^{-4}M
A4G4	AMO 1618 at concentration of 10^{-4}M in combination with GA_3 at concentration of 10^{-4}M
AMO 1618	2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride
C	Control treatment
E1	Ethephon at concentration of 0.1M
E2	Ethephon at concentration of 0.01M
E3	Ethephon at concentration of 0.001M
E3S3	combination of Ethephon at concentration of 0.001M and STS at concentration of 0.001M
FW	Fresh weight
G3	GA_3 at concentration of 10^{-3}M
G4	GA_3 at concentration of 10^{-4}M
G4I4	combination of GA_3 at concentration of 10^{-4}M and IAA at concentration of 10^{-4}M

G4I5	combination of GA_3 at concentration of 10^{-4}M and IAA at concentration of 10^{-5}M
G5	GA_3 at concentration of 10^{-5}M
G5I4	combination of GA_3 at concentration of 10^{-5}M and IAA at concentration of 10^{-4}M
GA_3	Gibberellic acid
I4	IAA at concentration of 10^{-4}M
I5	IAA at concentration of 10^{-5}M
I6	IAA at concentration of 10^{-6}M
IAA	Indole-3-acetic acid
LDW	Long day warm conditions
MT	Microtubule
PAR	Photosynthetically active radiation
PGR	Plant growth regulators
R/FR	Red/Far red
S1	STS at concentration of 0.01 M
S2	STS at concentration of 0.02 M

S3	STS at concentration of 0.001M
SDC	Short day cold conditions
STS	Silver thiosulphate
T3	TIBA at concentration of 10^{-3}M
T4	TIBA at concentration of 10^{-4}M
T5	TIBA at concentration of 10^{-5}M
TIBA	2,3,5-triiodobenzoic acid

Chapter 1

General introduction

1.1 Factors affecting stem elongation

Plants are sessile organisms that have special strategies to modify their development and growth responses to survive an ever changing environment. Indeed, the ability to react to complex environmental cues is crucial for both normal and adaptive development in a changing environment. This involves the correct incorporation of multiple external signals, such as temperature, light, gravity, wind, soil composition, water and nutrient availability etc., in all stages of development, from germination to flowering (Reid et al., 1991).

Temperature is an environmental factor that has a considerable influence on plant growth and development (Heggie and Halliday, 2005). The downstream pathways initiated and controlled by temperature cannot be considered in isolation. Each response draws upon integrated signals and downstream pathways, many of which are also regulated by other external cues, such as light (Heggie and Halliday, 2005). Temperature can be an important modifier of photoperiodic responses (Roberts and Struckmeyer, 1938), and the interaction between photoperiod and temperature strongly influences plant growth and development (Colidago and Brown, 1975). A study of the prairie ecotype of *Stellaria longipes* Goldie indicated that a combination of warm days and extended photoperiod best stimulates plant growth (Macdonald et al., 1984).

Stem elongation is a complex feature regulated by various environmental cues, such as photoperiod, quality and intensity of light, temperature, etc. These external factors, together with the internal factors, especially plant hormones, are believed to

affect the subapical meristematic region, which is the major site of cell multiplication and elongation, leading to stem elongation (Sachs, 1965; Kende et al., 1998). In many cases, stem growth does not occur until it is triggered by changes in environmental conditions, especially daylength and temperature, which results in rapid shoot growth or elongation (Metzger and Dusbabek, 1991). Elevated temperature was reported to enhance elongation growth in *Arabidopsis* hypocotyls and rosette internodes (Gray et al., 1998; Halliday and Whitelam, 2003).

Many of the temperature-controlled responses are mediated via the manipulation of endogenous plant hormone levels, which serve as powerful, yet adaptable controllers of plant development. Most common plant hormones that have significant effects on stem elongation include gibberellins, auxins and ethylene.

Gibberellins mediate a variety of light responses and are regulators of seed germination, stem growth, induction of flowering, pollen and fruit development, and numerous other plant responses (Sponsel and Hedden, 2004). Only a few GAs, including GA_1, GA_3, GA_4, GA_7 and GA_{97}, are considered to be growth-active whereas others are precursors in the GA biosynthesis pathway or are in inactive forms (reviewed by Sponsel and Hedden, 2004). In an early report on light-induced inhibition of stem growth, light was suggested to regulate the rate of stem growth through effects on gibberellin (GA) metabolism (Lockhart, 1958). GAs were reported to be involved in light-induced stem elongation of pea (*Pisum sativum*) (Barendse and Lang, 1972; Beall et al., 1996; Gawronska et al., 1995), canola (*Brassica napus*) (Potter et al., 1999), and long-stalked chick weed (*S. longipes*) (Kuperin et al., 2006c). The stem elongation of pea, cucumber (*Cucumis sativus*) and tobacco (*Nicotiana tabacum*) was proven to be more responsive to

applied GA in a low Red/Far Red (R/FR) compared to a high R/FR (Reid et al., 1990; Pierik et al., 2004). The likely role of GAs in photoperiodically controlled stem elongation and phenotypic plasticity of *S. longipes* has been discussed (Macdonald et al., 1986; Emery et al., 2001). Many studies have also been carried out to investigate the role of GAs in temperature-induced stem elongation. Moore (1979) suggested that there is a strong interaction between day and night temperatures and the endogenous levels of gibberellin within lily (*Lilium* sp.). In other application experiments, it was shown that the difference in stem elongation of plants grown under different temperature regimes were neutralized by GAs (Tangerås, 1979; Moe, 1990; Grindal et al., 1998a). Changes in endogenous levels of GA_1 may also mediate the altered stem elongation of pea in response to alternations of diurnal temperature (Grindal et al., 1998a).

Auxins are known to be closely linked to shoot growth because of their strong effect on elongation of isolated stem segments (Cleland, 1995). Auxin is believed to be synthesized in a wide rage of plant tissues (Bartel, 1997). Natural auxins include 4-chloroindole-3-acetic acid (4-Cl-IAA), indole-3-butyric acid (IBA) and phenylacetic acid (PAA) (Bartel, 1997). Indole-3-acetic acid (IAA), a naturally occurring auxin in plants, is known for its role in maintaining apical dominance by inhibiting development of auxillary buds, phototropism, gravitropism, fruit development, vascular tissue differentiation, stem elongation, cell division and so on (Leyser, 2003). Over the years, it has been shown that IAA regulates stem elongation response in pea (Behringer et al., 1990; Law and Davies, 1990; Yang et al., 1993), tobacco (Kraepiel et al. 1995), Arabidopsis (Steindler et al., 1999; Romano et al., 1993) and bean (*Phaseolus vulgaris*) (Bialek et al., 1983; Ortuno et al., 1990). It has been suggested that IAA is an essential

factor for temperature-induced stem elongation in pea seedlings (Yang et al., 1996). Gray et al. (1998) demonstrated that high temperature could promote the elongation of hypocotyl in light-grown *Arabidopsis thaliana* seedlings, and that this growth response partly depends on IAA. Furthermore, it was found that the observed thermoperiodic response on stem elongation in *A. thaliana* could be mediated through changes in the level of IAA (Thingnaes et al., 2003).

Ethylene is a gaseous plant growth regulator that is well recognized for its "triple response" in dark grown seedlings: inhibition of stem elongation, radial stem expansion and stem curvature (Basilevskaia et al., 1968). Ethylene is known for its effects in the inhibition (Lieberman, 1979; Abeles et al., 1992) or promotion (Raskin and Kende, 1984; Rijnders et al., 1997) of root and stem elongation. It has been suggested that ethylene might play a major role in plant responses to shading from neighboring vegetation in tobacco (Peirik et al., 2003; 2004), and sorghum (*Sorghum bicolor*) (Finlayson et al., 1998; 1999). However, it was suggested that a stimulation of elongation growth by ethylene may occur particularly at relatively low concentration (Fiorani et al., 2002). Similar to auxin, the effect of ethylene on stem elongation has also been controversial. While a large number of studies indicate that exogenous ethylene inhibits stem elongation, it has also been shown that ethylene promotes stem elongation in some plant species. Ethylene is essential for internodal elongation in deep water rice (Kende et al., 1998), other semi-aquatic plants (Jackson, 1985) and *S. longipes* (Emery, 1994; Emery et al., 1994; Kurepin et al., 2006; Walton et al., 2006). Although ethylene is quite important for plant growth and development, not much has been reported about the role of ethylene in temperature-induced stem elongation in plants.

At the cellular level, it has long been known that stem elongation is the result of cell elongation and/or cell division. The control of post-mitotic cell elongation is an important aspect of the process of morphogenesis, especially, the stem/internode elongation (Le et al., 2005). Cytoskeleton, composed of microtubules (MTs) and cellulose microfilaments, cell wall loosening proteins and internal pressure are known to play a principle role during diffuse growth of the plant cell (Cosgrove, 2005). MTs have been shown to orientate perpendicular to the direction of expansion and relate to cell elongation through their role in the orientation of the cellulose fibrils and cellulose fibril arrays (Cosgrove, 2005). The MTs orientate the cellulose complex along as they lie beneath the plant cell's plasma membrane and serve as tracks for membrane-associated cellulose synthases (rosette structures) to travel along (Lloyd, 2006). As the synthase moves, it deposits cellulose microfibrils into the adjacent cell wall. Novel microfibrils are deposited along MTs and in parallel with the pre-existing cellulose microfibrils, guided by the arrangement of proteins in both the cell wall and matrix plasma lemma (Lloyd, 2006). The cell shape is partially regulated by the orientation of MTs. The longitudinal organization of microtubules is found in the cells at the base of a plant where the cell elongation ceases, preventing the cell from elongation. Transversely aligned microtubules, on the other hand, are found in cells in the middle and at the top of the plant where the cell elongation and expansion take place. This orientation of MTs allows the cell to expand longitudinally (Erhard and Shaw, 2006). Direction of cell expansion and the orientation of MTs is regulated by plant hormones, such as gibberellins (GAs), brassinosteroides, cytokinins, and auxin. Many studies have coupled the effect of plant hormones on the reorientation of MTs and cellular structures, but little is known about

the correlation between environmental signals, plant growth regulators, and developmental and structural changes. It is, therefore, important to assess morphological and structural changes of plants in responses to different temperatures, and examine how key hormones, including ethylene, auxin or gibberellins (GA_3), regulate the temperature-induced stem elongation. This study illustrates the influential role that temperature plays throughout growth of *Stellaria longipes* and the close interaction between temperature, hormone signaling and cellular structures.

1.2 The plant system

Stellaria longipes is a polymorphic herbaceous perennial species that shows a large degree of phenotypic plasticity reflected in morphological changes, such as stem elongation, leaf shape and flower number (Chinnappa et al., 2005). Stem elongation provides *S. longipes* genotypes with the fitness to survive in their respective environments. The levels of stem elongation of this species differ among ecotypes and the most distinct morphological differences are exhibited by the alpine and prairie ecotypes (Chinnappa et al., 2005). The alpine ecotype grows in tundra habitat (elevation of 2,453 m) where it is exposed to a high wind stress, lower temperature and normal light irradiance. A short stem helps this ecotype to survive in that severe weather condition. The taller prairie ecotype, on the other hand, grows in a grass land habitat (elevation of 1,310 m) where it is under intense competition from other plant species. The inherited plasticity of this ecotype allows survival in such a competitive environment (Emery et al., 1994). Prairie and alpine ecotypes represent two extreme cases of stem elongation response and they are ideal candidates for the comparative study of stem elongation.

1.3 Research objectives

Many studies have investigated the effect of endogenous/exogenous ethylene (Emery et al., 1994; Kathiseran et al., 1998; Miranda et al., 2001; Kurepin et al., 2006a), gibberellins (MacDonald et al., 1986, Emery et al., 2001; Kurepin et al., 2006a; Kurepin et al., 2006c), auxins and cytokinins (Kurepin et al., 2008) on the stem elongation of *S. longipes*. The effects of environmental conditions, including light quality and irradiance (Alokam et al., 2002; Kurepin et al., 2006b), wind stress (Emery et al., 1994) and photoperiod (Walton et al., 2006), have been carefully investigated. However, the effect of temperature on stem elongation has not been thoroughly studied except for one study by Macdonald et al. (1984), in which the effect of temperature on a prairie ecotype was examined. Also, the possible interaction between temperature and phytohormones in the regulation of stem elongation has not been investigated. The experiments carried out in this study examined the morphological, physiological, and histological aspects of stem elongation of alpine and prairie ecotypes of *S. longipes*. The following questions were addressed:

- How do alpine and prairie ecotypes respond to different temperature regimes?
- What are the major structural and cellular changes of alpine and prairie ecotypes to temperature?
- What is the possible role of auxin (IAA), ethylene, gibberellic acid (GA_3) in the response of alpine and prairie ecotypes to temperature?

In chapter 2, I present a study which aimed to (i) examine the effects of temperature and photoperiod on the stem elongation of alpine and prairie ecotypes of *S. longipes* to confirm the importance of temperature on the stem elongation, and (ii) examine the effect of temperature on the morphological changes of both ecotypes.

Chapter 3 focuses on elucidating the morphological and histological aspects that underlined the differences in temperature-induced stem elongation of alpine and prairie ecotypes of *S. longipes*. The study was initiated in an attempt to better understand the effect of two contrasting temperature regimes on the stem elongation of alpine and prairie ecotypes, and to use that as a system to determine the cellular characteristics that involve in the possible differences between the two ecotypes on the basis of some cellular characteristics. Chapter 4 examines the effects of changing temperature regimes on ethylene evolution by the alpine and prairie ecotypes of *S. longipes* in an effort to further understand the role of key plant growth regulators in plants adapted to different environmental conditions. Chapter 5 was to indirectly uncover the role of gibberrelic acid and auxin (IAA) in the response of alpine and prairie ecotypes of *S. longipes* grown under different temperature regimes by exogenously applying plant growth regulators on intact plants and detached segments.

The importance of this project is to provide a better understanding of temperature effects on stem elongation of alpine and prairie ecotypes of *S. longipes* and how it may control adaptive morphological plasticity of these two distinct ecotypes. Hence, results from this study are important for the elucidation of the evolutionary significance of plasticity of *S. longipes*. As a result, a better understanding of physiological and developmental controls of stem elongation response of alpine and prairie ecotypes of *S. longipes* could be achieved.

Chapter 2

Temperature effects on the stem elongation of alpine and prairie ecotypes of *Stellaria longipes*

2.1 Introduction

Temperature and photoperiod are two primary environmental factors that control growth and development of plants. Alterations in light quality, quantity and duration provide plants with information which results in different morphological responses. Prolonged photoperiod promoted stem elongation of catchfly (*Silene armenia* L.), corncockle (*Agrostemma githago* L.), and spinach (*Spinacia oleracea* L.) (Jones and Zeevaart, 1980; Talon and Zeevaart 1990; Talon et al., 1991). Similarly, it was reported that increasing daylength was positively correlated to increased stem elongation of *Stellaria longipes* (Walton et al., 2006). Reduced irradiance of photosynthetically active radiation (PAR) and R/FR ratio can also promote stem elongation (Ballare et al., 1991; Franklin and Whitelam, 2005). The prairie ecotype of *S. longipes* showed very significant increase in stem elongation in response to low R/FR ratio relative to that under a normal R/FR ratio. However, the alpine ecotype showed only a slight increase in stem elongation under R/FR enrichment (Alokam et al., 2002; Chinnappa et al., 2005; Kurepin et al., 2006b). In addition to light, temperature also has complex and profound effects on plant growth.

Plant growth can occur over a wide range of temperatures, which can be defined at three basic levels: (i) a minimum temperature below which no growth occurs, (ii) an optimum temperature at which the greatest growth occurs, and (iii) a maximum temperature above which no growth occurs. Growth rate increases above the minimum

temperature until an optimum is reached, then declines until the maximum temperature is reached. The minimum, optimum and maximum temperatures vary among species (Heggie and Halliday, 2005). Most plants do not respond in the same way to temperature at all stages of growth. In fact, the same warm temperatures may be detrimental to growth as the plants become mature. Plants in a vegetative stage of growth generally have a warmer temperature optimum than those in a reproductive stage. Also, different parts of the same plant may have different optimum temperatures for growth (Heggie and Halliday, 2005).

Temperature effects on stem elongation have been widely studied, given that the global temperature is increasing (Heggie and Halliday, 2005). Plant morphology can be shaped quite dramatically by altering day and night temperatures (Myster and Moe, 1995). Differences in day and night temperatures were shown to affect stem elongation in a wide range of plant species, including tomato (*Lycopersicon esculentum*) (Went, 1944), chrysanthemum (*Chrysanthemum* sp.) (Karlsson and Heins, 1986; Carvalho et al., 2002), chili pepper (*Capsicum annum*) (Dorland and Went, 1947), and lily (*Lilium* sp.) (Roh and Wilkin, 1973; Erwin et al., 1989). In many cases, stem growth does not occur until it is triggered by changes in environmental conditions, especially day length and temperature (Metzger and Dusbadek, 1991). Elevated temperature enhances elongation growth in Arabidopsis hypocotyls and rosette internodes in responsive vegetative tissue (Gray et al., 1998; Halliday and Whitelam, 2003). In some species, rapid stem elongation is the result of internodal expansion and stem elongation after an inductive treatment (Zeevaart, 1983; Metzger, 1987). In addition, many of the temperature-related developmental pathways are intimately linked to light signaling.

Knowledge on the mechanisms underlying the temperature-induced elongation in plants, however, is fairly limited. It increases general understanding of the interaction between environmental factors and stem elongation (Stavang et al., 2005). Earlier work on a prairie ecotype of *Stellaria longipes* showed that temperature had a significant effect on its stem elongation (Macdonald et al., 1984). This study aimed to (i) examine the effects of different temperature regimes and photoperiod on the stem elongation of alpine and prairie ecotypes of *S. longipes*, and (ii) examine the effect of temperature on the morphological changes of both ecotypes. It is hypothesized that temperature affects stem elongation of the alpine and prairie ecotypes differently. Prairie ecotype would have a wider range of response to temperature where plants can grow and respond vigorously. Alpine ecotype, on the other hand, will not have such plasticity to respond to similar temperature treatments. Not only they would elongate at different magnitude, but also attain differences in other morphological traits.

2.2 Materials and methods

Plant material

Two ecotypes of *S. longipes* were collected in the Kananaskis Country, Alberta. One genotype representing alpine ecotype from the summit of Plateau Mountain (2453 m elevation) and the second genotype representing prairie ecotype from the Chain Lakes area (1310 m elevation) in Southern Alberta (50°16'N and 114°31'W) were used. All plants were potted in 3 x 4 inche pots containing peat moss, sand and Terra green (2:1:1). The plants were maintained in the greenhouse before placing in growth chambers under short day cold (SDC) conditions (8h photoperiod and 8°/4°C, day/night) for a minimum

of 60 days to simulate winter conditions (Macdonald et al., 1984). Plants were transferred to long day warm (LDW) conditions (16h photoperiod and 22°/16°C, day/night) for 3 days so that plants could recover after a long winter period. The apical meristem is the site of temperature perception for growth and development. In addition, the plants must reach a certain stage of growth before the apical meristem is sensitive to temperature because the cells of the meristem must be metabolically active to perceive the temperature stimulus (Heggie and Halliday, 2005). Therefore, both alpine and prairie ecotypes were placed under LDW conditions after 60 days in SDC so plants can recover from the cold winter and acclimatize to the new environment. Plants were then transferred to different chambers with assigned temperature regimes under the light irradiance of ~120 μmol m^{-2} s^{-1} and the photoperiod of 8h or 16h.

Growth chambers

To study the response of alpine and prairie ecotypes to varied temperature regimes, a minimum of four growth chambers (Conviron, Winnipeg, Canada) were required. The conditions of these chambers were set depending on the purpose of the experiment. The light irradiance and Red/Far red (R/FR) ratio were maintained as ~120 μmol m^{-2} s^{-1} and 1.76 ± 0.02, respectively.

Growth measurements

Ramets of alpine and prairie ecotypes were selected randomly, marked at day zero with a permanent marker and transferred into assigned chambers. The total stem length of the ramets of both ecotypes was measured on day 0, 5, 8, 11, 14 and 21. The 2nd pair of

leaves from the apex of each plant were collected and pressed for measurements of leaf area. The second internode from the shoot apex was also taken for measurement of internode length. Fresh weight of the whole shoot was also recorded.

Experimental designs

Stem elongation of alpine and prairie ecotypes in response to different temperature and photoperiod regimes

To observe and measure the effects of varying temperatures and photoperiods on stem elongation and leaf area of alpine and prairie ecotypes, they were kept in four different temperature regimes in combination with two photoperiods as following:

Treatment	Temperature (°C)			Photoperiod
	(8:00 am – 4:00 pm)	(4:00 pm – 12:00 pm)	(12:00 pm – 8:00 am)	(hours)
1	16	10	10	8
2	16	16	10	16
3	24	10	10	8
4	24	24	10	16
5	8	10	10	8
6	8	8	10	16

Effects of different temperature combinations on stem elongation of the alpine and prairie ecotypes

The effects of heat on the stem elongation of both ecotypes grown under the same photoperiod condition were studied by maintaining both ecotypes in growth chambers with combinations of temperature as following:

Treatment	Temperature (°C)		
	(8:00 am – 4:00 pm	(4:00 pm – 12:00 pm)	(12:00 pm – 8:00 am)
1	28	28	16
2	28	16	16
3	28	28	10
4	28	10	10
5	28	28	22

Light: PAR: ~ 120 μmol m^{-2} s^{-1}, photoperiod: 16h

Stem elongation of alpine and prairie ecotypes in response to different temperature regimes

Alpine and prairie ecotypes were placed in growth chambers with conditions, as follows, to study the effects of different temperature regimes on plant growth under the same photoperiod condition:

Light: PAR: ~ 120 μmol m^{-2} s^{-1}, photoperiod: 16h

Treatment	Temperature (°C)		
	(8:00 am – 4:00 pm	(4:00 pm – 12:00 pm	(12:00 pm – 8:00 am)
1	10	10	4
2	16	16	10
3	22	22	16
4	28	28	22
5	34	34	28

Data analysis

Each experiment was conducted three times. Data were analyzed by means of a three-way repeated measures analysis of variance (ANOVA). Honestly significant

difference (HSD) Tukey test was used to determine differences between temperature treatments (SAS Institute Inc., 2004). The data are reported as mean ± standard error of the mean and differences are considered significant at $P < 0.05$.

2.3 Results

Stem elongation of alpine and prairie ecotypes in response to different temperature and photoperiod regimes

No significant difference in stem elongation of the alpine and prairie ecotypes grown under different temperature regimes and 8h photoperiod was observed although higher temperature regimes, to some extent, promoted stem elongation (Fig. 2.1 A, C). Differences in the responses of these two ecotypes were more significant when they were grown under 16h-photoperiod than under 8h-photoperiod (Fig. 2.1 B, D). The response was much more remarkable as plants were grown under higher temperature regimes. Warmer temperature better stimulated stem elongation of both ecotypes.

There was no difference in stem elongation of the alpine ecotype grown under different temperature regimes when plants were grown under 8h-photoperiod (Fig. 2.1A). Significant difference in stem elongation was seen only between plants grown under the lowest (8°/10°C) and highest (24°/10°C) temperature regimes (Fig. 2.1A). Stem elongation was significantly different among the alpine plants grown under different temperature regimes with 16h-photoperiod (Fig. 2.1B). As temperature increased, the elongation ability of plants also increased. The highest temperature regime (24°/10°C) resulted in the best growth of the alpine plants (15 fold increase in growth). Growth was

significantly retarded under the lowest temperature regime (8°/10°C) (only 5 fold increase in growth) (Fig. 2.1B).

Under both photoperiod conditions, the alpine ecotype did not start elongating until day 5. Growth of the alpine ecotype can be divided into three stages: (i) day 0 to day 8: lag phase, plants prepared themselves to elongate, (ii) day 8 to day 14: considered the rapid elongation stage, (iii) day 14 to day 21: the stable developmental stage when the plants elongated slowly and the growth rate declined.

As for the prairie ecotype, there was no significant difference in stem elongation between plants grown under 8°/10°C and 16°/10°C and 8h photoperiod (Fig. 2.1C). Plants grown under 24°/10°C elongated the most (Fig. 2.1C). Rapid elongation stage differed among plants grown under different temperature regimes. Under 8°/10°C and 16°/10°C, the rapid elongation stage was from day 8 to day 11, while under 24°/10°C the rapid elongation was from day 5 to day 11 (Fig. 2.1C).

Significant difference in stem elongation among the prairie plants grown under different temperature regimes and 16h photoperiod was obtained. The highest temperature best stimulated stem elongation. Temperature regime of 24°/10°C favored stem elongation whereas that of 8°/10°C significantly retarded it (Fig. 2.1D). The prairie plants started elongating right after transfer from LDW to assigned temperature treatments. Under 8°/10°C and 16°/10°C, the rapid elongation stage was from day 8 to day 11, whereas the rapid elongation stage of plants grown under 24°/10°C was from day 5 to day 14. After rapid elongation stage, stem elongation slowed down noticeably.

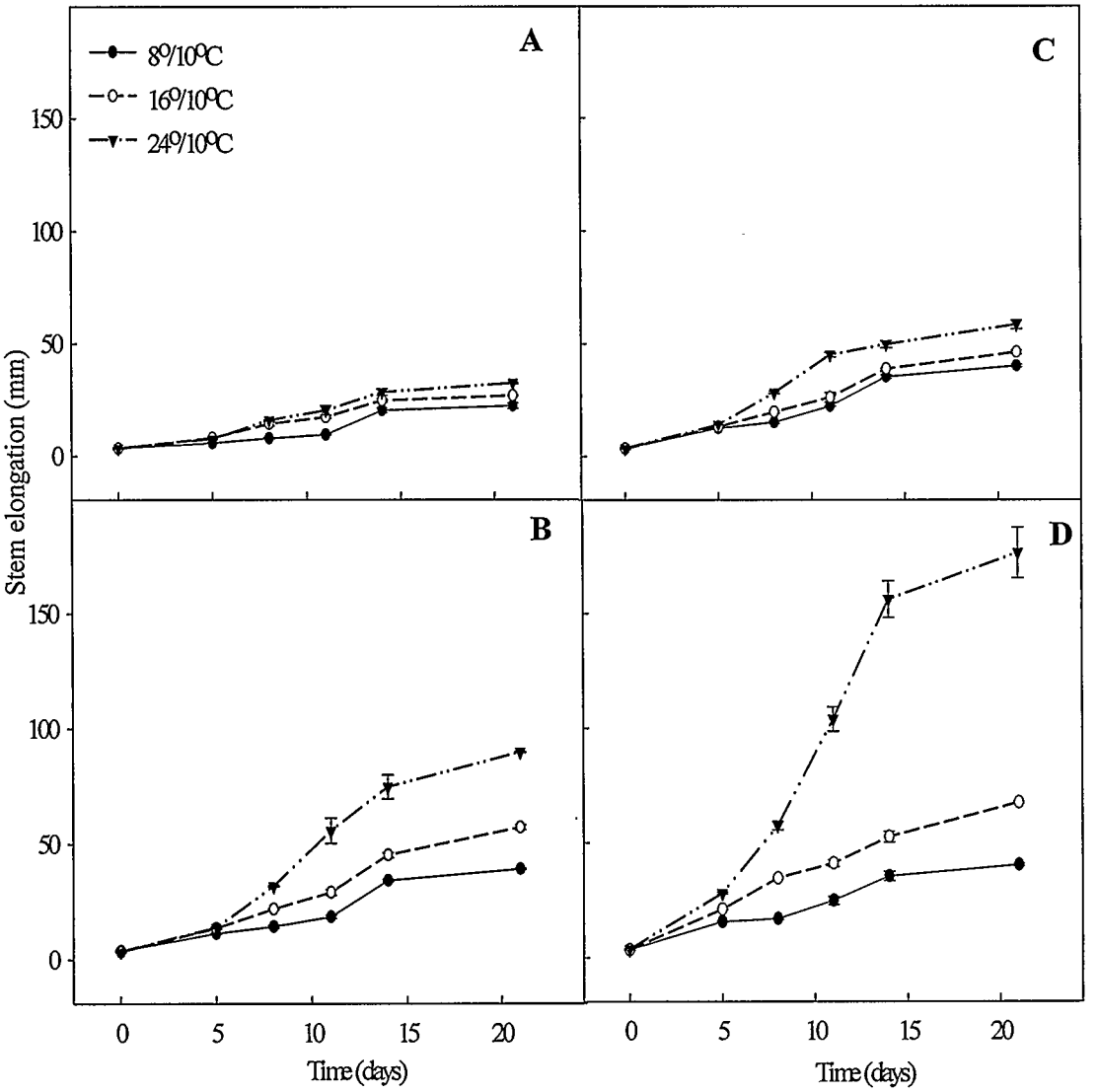

Figure 2.1. Stem elongation of the alpine (A and B) and prairie (C and D) ecotypes of *Stellaria longipes* grown under three different temperature regimes in combination with 8h (A and C) and 16h (C and D) photoperiod. The error bars indicate standard error of the mean.

Effects of different temperature combinations on stem elongation of the alpine and prairie ecotypes

When the alpine ecotype was provided with different amount of heat created by different combinations of temperature regimes, stems elongated more as the temperature increased. Plants were tallest under 28°/22°/22°C and shortest under 28°/10°/10°C. There was significant difference between the highest and lowest temperature treatments (Fig. 2.2A). Interestingly, there was no difference in stem length between plants grown under the hottest and the second hottest temperature regimes, implying a limited response of the alpine ecotype to warm temperature treatment.

Similar to the response seen in the alpine ecotype, the higher the temperature, the more the prairie ecotype grew. Plants were able to accrue the heat that is provided and responded accordingly (Fig. 2.2B). Surprisingly, significant differences were recorded between plants grown under the hottest and the second hottest temperature regimes, indicating special response of the prairie ecotype to increased temperature. Therefore, it seemed that the amount of heat that was provided significantly promoted stem elongation of the alpine and prairie ecotypes. However, these two ecotypes responded differently to the same temperature regimes, suggesting that the prairie ecotype is more sensitive to temperature, especially higher temperature than the alpine ecotype and the prairie is more plastic to increase in temperature than the alpine.

Stem elongation of alpine and prairie ecotypes in response to different temperature regimes

Experiments on the effect of different temperature regimes on the stem elongation of alpine ecotype showed that under the same light conditions, higher temperatures would favor stem elongation (Fig. 2.3A). There was a range of temperatures that plants could respond best, and they were from 16°/10°C (day, night) to 28°/22°C (day, night). As temperature went beyond (34°/28°C, day, night) or below (10°/4°C), stem elongation was retarded. Stem elongation response of the alpine ecotype can be divided into three groups: plants grown under cool temperatures (10°/4°C and 16°/10°C), warm temperatures (22°/16°C and 28°/22°C – group 2) and hot temperature (34°/28°C – group 3). There was no difference in stem elongation among plants grown under the same group of temperature regimes. Plants grown under 22°/16°C and 28°/22°C temperature regimes were taller than those grown under cool or hot temperature regimes. The rapid elongation stage of plants grown under warm conditions was from day 5 to day 14. The rapid elongation stage of plants grown under cool conditions was from day 5 to day 11. Hottest temperature regime (38°/32°C) inhibited stem elongation of the alpine ecotype (Fig. 2.3A).

As for the prairie ecotype, under the same light conditions, plants also elongated more when grown under warm temperature regimes than others. There was significant difference in stem elongation among treatments. Plants grown under 28°/22°C were the tallest, whereas plants grown under 10°/4°C were the shortest. As temperature went beyond 28°/22°C, stem elongation was also inhibited. The prairie ecotype started elongating right after the transfer from LDW conditions. Rapid elongation stage was from day 5 to day 14 for plants grown under warm conditions. For plants grown under cool and hot conditions, the rapid stage of elongation was from day 8 to day 14 (Fig. 2.3B).

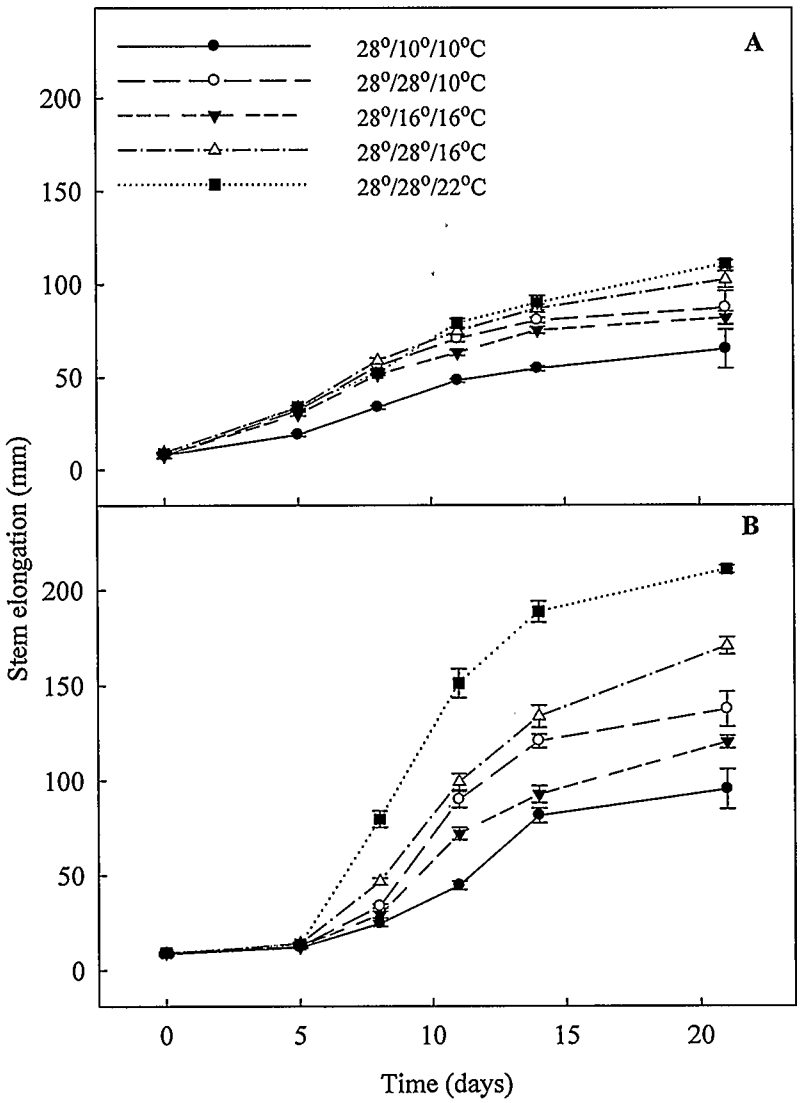

Figure 2.2. Cumulative stem elongation of alpine (A) and prairie (B) of *Stellaria longipes* grown under five different temperature combinations from day 0 to day 21. The error bars indicate standard error of the mean.

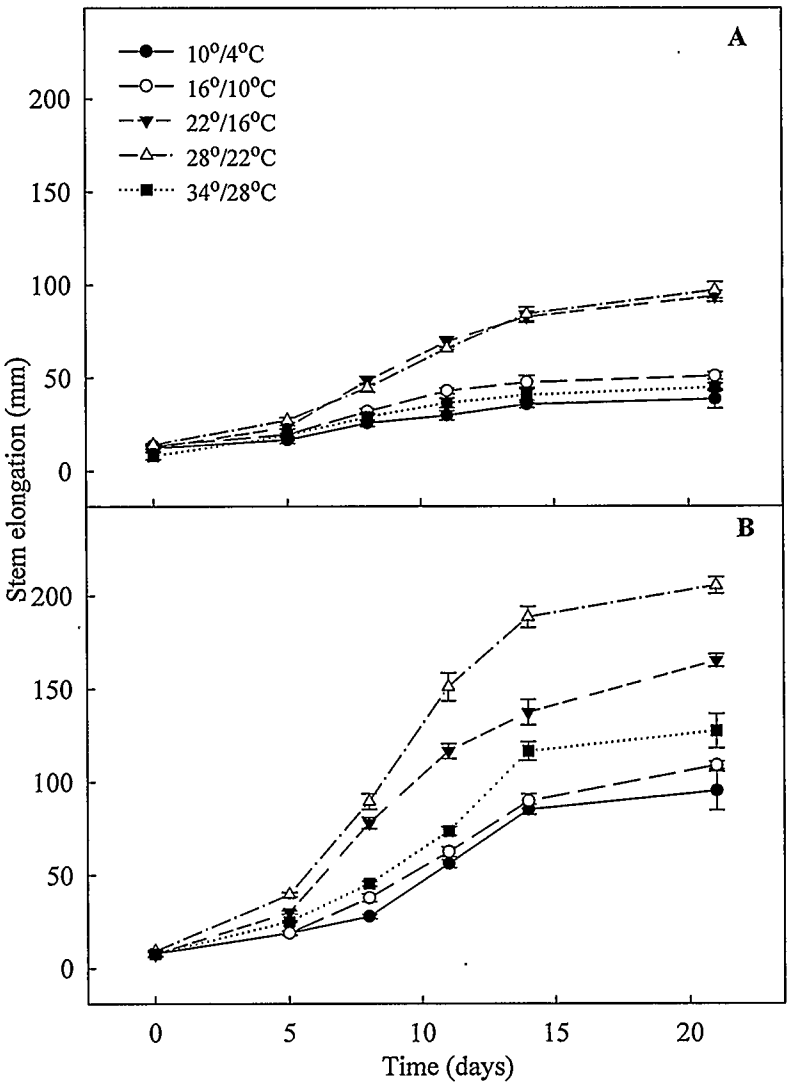

Figure 2.3. Cumulative stem elongation of alpine (A) and prairie (B) of *Stellaria longipes* grown under five different temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean.

In addition to stem length, some morphological changes of plants grown under different temperature regimes, including leaf area, fresh weight and internode elongation, were also recorded.

As for the alpine ecotype, the internodes were longer when plants were grown under warmer temperature regimes. There was significant difference in internode elongation among treatments. Internodes from plants grown under warm temperatures were the longest. Internode started elongating shortly after transfer to assigned temperature treatments. There was no difference in internode elongation between 28°/22°C and 22°/16°C (Fig. 2.4A).

A similar response was observed in the prairie ecotype. Internodes started elongating after transfer to different temperature regimes. Rapid stage of elongation was from day 0 to day 14. After day 14, internode elongation was slowed down to day 21. There was significant difference in internode elongation among treatments. The internodes from plants grown under 28°/22°C were the longest and internodes from plants grown under 10°/4°C were the shortest. Temperature regimes of 34°/28°C inhibited the growth of plants and internodes (Fig. 2.4B).

Shoot weight of the alpine ecotype increased with increased temperatures. There were significant differences in shoot weight among treatments. Shoot was heaviest when plants were grown under warmer conditions and lightest under cooler temperatures (Fig. 2.5A). As for the prairie ecotype, there was significant difference in fresh weight of plants grown under different temperature regimes. Plants grown in warmer temperatures were heavier than plants grown under cooler temperatures. Too hot or too cold

temperature retarded the growth of plants, resulting in lower fresh weight of the shoots (Fig. 2.5B)

There was significant difference in leaf area of the alpine plants grown under different temperature treatments (Fig. 2.6A). Leaves of plants developed under cooler temperatures were smaller than those grown under warmer temperatures. There was also difference in the shape of the leaves. Leaves developed under cooler temperature were lanceovate whereas those of plants grown under warmer temperatures were elongated. Leaves rapidly expanded as soon as plants were transferred to the assigned temperature treatments. Once stem elongation was slowed down in plants, leaves did not expand very much.

There was significant difference in leaf area of the prairie plants grown under different temperature treatments. Leaves developed under warmer temperature conditions were much bigger than those of plants grown under cooler temperatures. Leaves started expanding as soon as plants were transferred to different temperature treatments. The rapid expansion stage was from day 0 to day 8. Leaves slowly expanded right after transfer and expansion was somewhat stopped by day 11 (Fig. 2.6B).

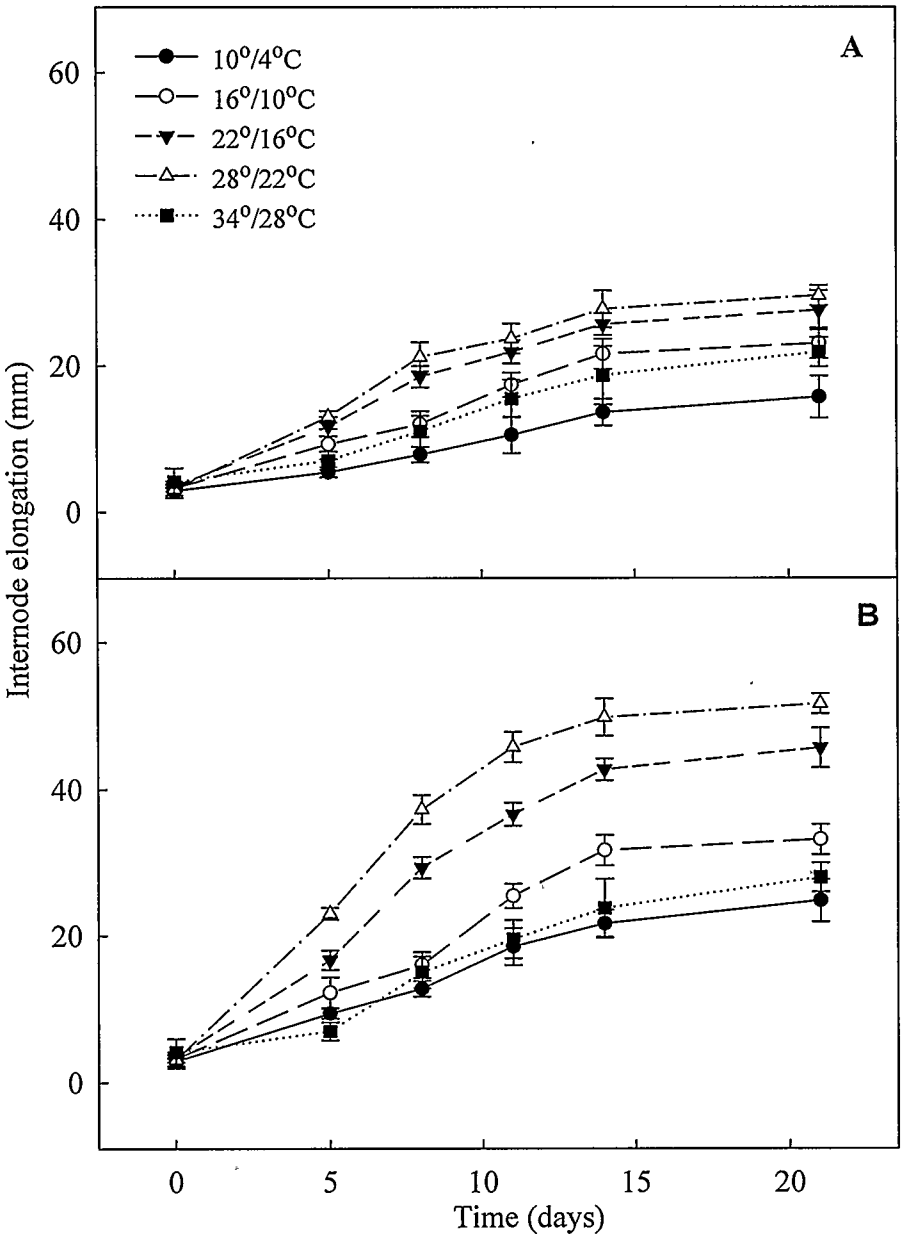

Figure 2.4. Cumulative internode elongation of alpine (A) and prairie (B) ecotypes grown under 5 different temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean.

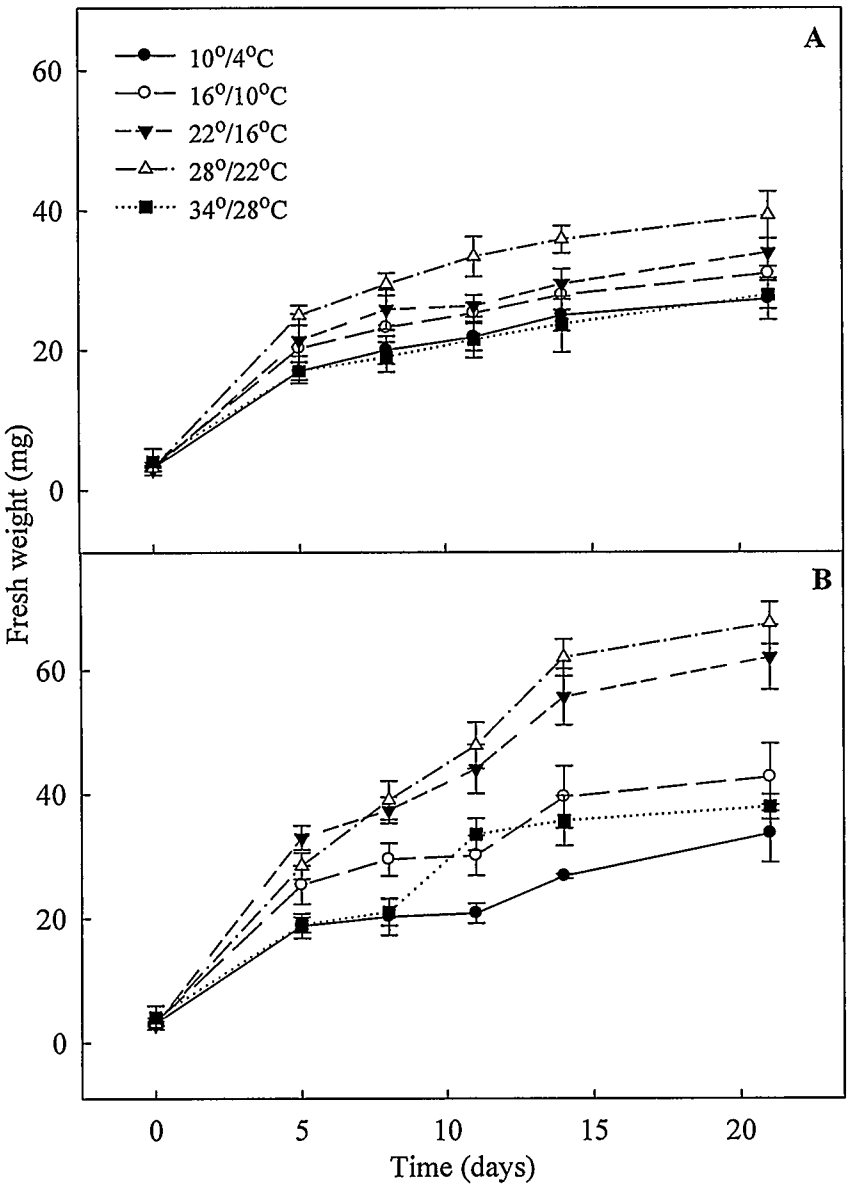

Figure 2.5. Cumulative fresh weight of alpine (A) and prairie (B) ecotypes grown under 5 different temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean.

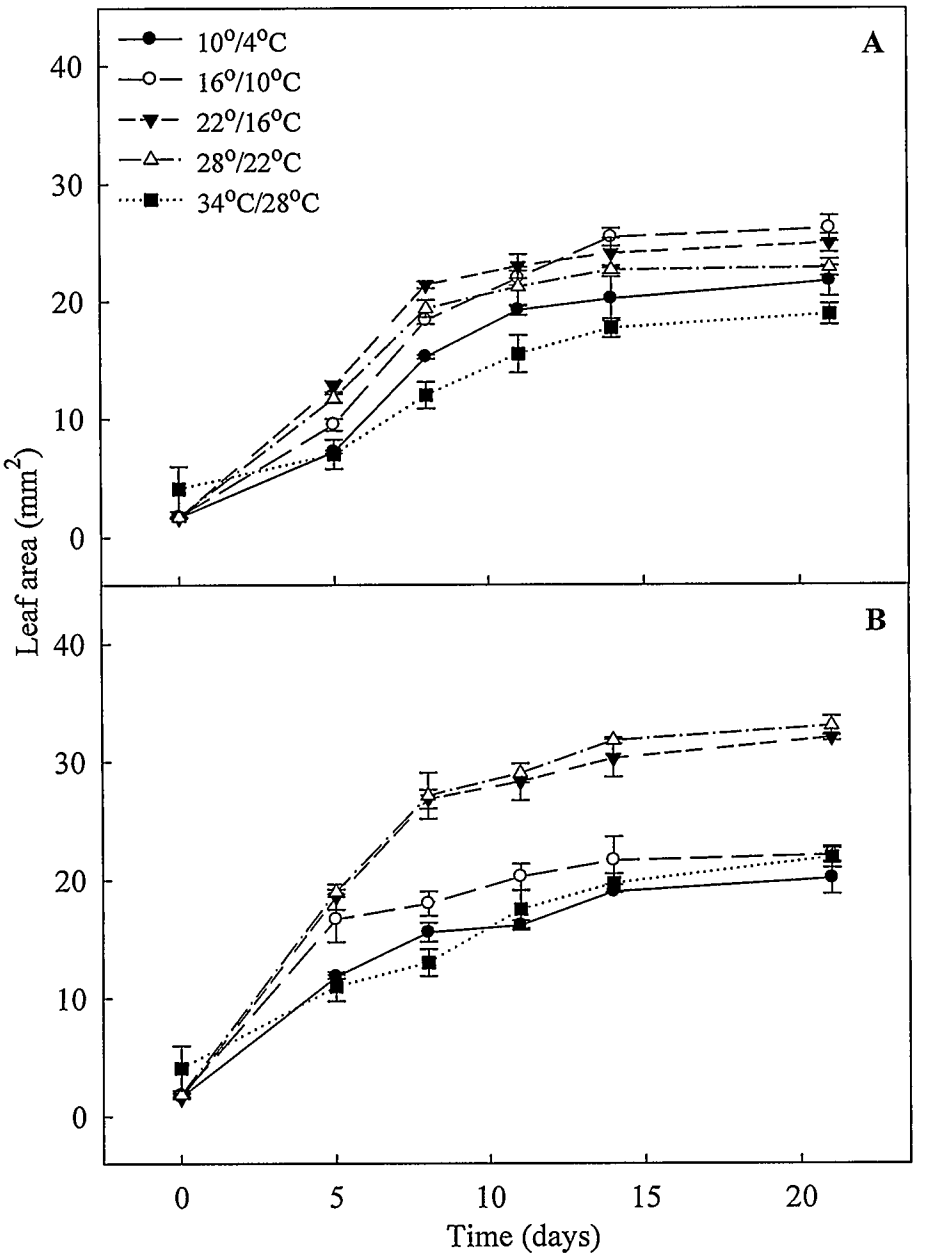

Figure 2.6. Leaf area of alpine (A) and prairie (B) ecotypes grown under 5 different temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean.

2.4 Discussion

Temperature is one of the most influential environmental factors on plant growth. The magnitude and nature of the thermo-morphogenic effects vary between plant species, as well as with timing and duration of the temperature fluctuation (Erwin and Heins, 1995).

Stem elongation response of the alpine and prairie ecotypes to temperature is affected by photoperiod. Over the years, it has been reported that quality of light (R/FR ratio) and photoperiod promoted stem elongation of *S. longipes* (Alokam et al., 2002; Kurepin et al., 2006b, c; Walton et al., 2006). However, it is shown, in my study, that temperature is an important factor in inducing and maintaining the stem elongation responses of the alpine and prairie ecotypes of *S. longipes* (Fig. 2.1). How does light itself interact with temperature in the control of elongation and expansion in plant development is still a question to be answered. Weinig (2000) showed that temperature has a major impact on elongation responses to low R:FR ratio light in the annual weed *Abutilon theophrasti* (velvetleaf) by altering hypocotyl elongation, suggesting that temperature and light may be acting synergistically in this response. Results from the first experiment showed that a combination of higher temperature and long photoperiod promoted stem elongation of alpine and prairie ecotypes. Walton et al. (2006) also reported that increased day length was positively correlated to increased stem elongation of both ecotypes. Macdonald et al. (1984) suggested that temperature is the most important factor for the induction of stem elongation of one genotype of *S. longipes*, while photoperiod exerts a lesser effect. In addition, it was suggested that light appears to

have a repressive effect on internode elongation stimulated by elevated temperature during vegetative development (Mazzella et al., 2001; Halliday and Whitelam, 2003).

Plant height is simply the sum of the lengths of each of the internodes. My results showed that there was a strong correlation between the internode and stem elongation. Internodes manifested somewhat the same level of elongation as the stem in response to different temperature regimes. The rate of node development is driven primarily by short and long term average temperature (Karlsson et al., 1989). Internode elongation, which is an indirect measure of stem elongation, shows a curvilinear response to temperature, increasing as temperature increases to an optimum, then decreasing if temperature becomes too high or too low. Internode length in many plants is greatly influenced by diurnal temperature fluctuation (Erwin et al., 1994). Results of my experiments showed that the prairie ecotype responded more rapidly to changes in temperature. Indeed, the response does not appear to have much of a residual effect, that is, both alpine and prairie plants respond to the current environmental regime with little lag or long term carry over.

The magnitude and nature of the temperature-induced stem elongation are influenced by plants species and cultivars. It is very clear that the alpine and prairie ecotypes have different responses to temperature regimes. Curiously, the alpine ecotype did not seem to have the ability to respond to high temperature regimes, suggesting that these two ecotypes have different strategies to cope with changes in temperature. Since the alpine ecotype is rarely exposed to such a high temperature regimes, the plants might have lost their ability to promote stem elongation under these conditions. It is possible that increased elongation is an adaptive response to high temperature. Stem elongation elevates the photosynthetic and meristematic tissues away from the heat-absorbing soil

and may allow the plant to take better advantage of the cooling effect of moving air. This response could potentially provide plants with the adaptive advantage under unfavorable conditions, which could be referred to as "heat avoidance response".

The timing and length of temperature changes influence the response to photo-skotoperiod temperature responses (Berghage, 1998). Stem elongation is greatest during the end of the night and the beginning of the day, decreasing during the day and increasing again during the night (Erwin and Heins, 1995). Our results suggested that regardless of the time, the plants grew more when more heat was provided.

Leaf area of potato (*Solanum tuberosum*) plantlets (Kozai et al., 1995) and *Brassica* transplants (Bakken and Flones, 1995) was reduced when plants were grown with low day temperature. Leaf area of both the alpine and prairie ecotypes were reduced under cooler temperature regimes and promoted under warmer temperature regimes. Interestingly, there was not much difference in leaf area among treatments, however, the shapes of leaves did changed, implying that phytohormones, especially auxin, might be involved in this response.

Lower day temperature and reduced leaf cholorophyll have been considered responsible for the frequently reported reduction in weight of plants grown in unfavorable conditions (Berghage, 1998). Grimstad (1993) reported reduced plant dry weight in both cucumber and tomato in response to a low temperature pulse. Fresh weight of both the alpine and prairie plants was also significantly reduced when plants grew under cooler temperature regimes.

When plants are exposed to stress conditions, i.e. a temperature above or below normal physiological range, they exhibit various responses and their photosynthetic

performance is affected as well (Lichtenthaler, 1996). My results suggested that optimum temperature for growth of the alpine ecotype was from 16°C to 22°C whereas that of the prairie ecotype was from 22°C to 28°C. The alpine ecotype was more sensitive to cold temperature than the prairie ecotype, whereas the prairie ecotype was more sensitive to warm temperature than the alpine. There is a general consensus that the optimum temperature for photosynthesis exhibited by a plant species has been genetically and physiologically adapted (Berry and Bjorkman, 1980). Yet, too cold or too warm temperature retarded the growth of both alpine and prairie ecotypes of *S. longipes*. Plants might exhibit a different degree of plasticity with respect to the temperature of photosynthesis (Georgieva and Lichtenthaler, 2006).

In conclusion, the same temperature regime exerted different effects on the growth of alpine and prairie plants. Variation in stem elongation between the two ecotypes grown under the same conditions is most likely due to differences in their ability to respond to specific temperature regimes. The difference in elongation ability of these two ecotypes provides an ideal comparative system to study the cellular structures or physiological aspects which underlie their stem elongation. In the real world, plants have to respond to changes in the external environment, but they also have to maintain development when conditions fluctuate.

Chapter 3

Differences in cellular structure and stem elongation of alpine and prairie ecotypes of *Stellaria longipes* grown under contrasting temperature regimes

3.1 Introduction

Growth of plants is strictly controlled externally by the environment and internally by their developmental program (Fry, 1988). Stem elongation, which helps a plant to adjust to various environmental conditions, is a complex trait controlled by various environmental cues, such as photoperiod, light quality and irradiance, temperature, and other factors. These external factors, acting in concert with plant growth hormones are believed to affect the subapical meristematic region, which is the major site of cell multiplication and elongation, leading to stem elongation (Sachs, 1965). In many cases, stem growth does not occur until it is triggered by changes in environmental conditions, especially day length and temperature, which results in rapid shoot growth or elongation (Metzger and Dusbadek, 1991). In some species, rapid stem elongation is the result of internodal expansion after an inductive treatment (Zeevaart, 1983; Metzger, 1987). At the cellular level, stem elongation resultes from cell division and elongation. During the rapid growth phase, cell elongation is determined by different factors, such as the microtubular cytoskeleton, cell wall materials, cell wall loosening proteins and turgor pressure (Cosgrove, 2005). Of all, microtubules (MTs) play a major role in cell elongation since they regulate the direction of expansion in plant cell walls (Cosgrove, 2005).

Stellaria longipes is a model system that has been used to study the physiological, genetic and molecular aspects of stem elongation plasticity (Chinnappa et al., 2005). The alpine and prairie ecotypes represent two extreme cases of stem elongation plasticity response (Chinnappa et al., 2005). Although there have been many studies on stem elongation of crop species, studies using wild species are fewer in number. Earlier work demonstrated that both photoperiod and temperature are important in stem elongation plasticity of a prairie genotype (Macdonald et al., 1984; Chuong et al., 2001). In this study, we focused on the temperature effects on stem elongation plasticity in two ecotypes. Alpine and prairie ecotypes of *S. longipes* were exposed to two different temperature regimes and some developmental and cellular characteristics were recorded. It was hypothesised that the alpine and prairie ecotypes would respond differently to contrasting temperatures due to differences in cellular changes.

3.2 Materials and methods

Plant material

Plant material was prepared as described in Chapter 2.

Histochemical detection and observation of cell length/width

Increments in growth of ramets in the assigned temperature regimes (10°/4°C and 28°/22°C, day and night) were measured from the reference point (previously created with a permanent marker on day 0) to the shoot apex by a ruler to the nearest mm at different time points of the growth period (day 0, 5, 8, 11, 14 and 21). The first visible internodes below the shoot bud were also collected to record internode elongation. Existing internodes at the time of transfer were not used for the elongation study as they

had began to develop under short day cold conditions and were less responsive to experimental manipulation. Hence, internodes that were formed at the time of transfer (first internodes from the top) were chosen for morphological and histological studies. The elongation and histology of the same internode was followed from the first day of transfer (day 0) until the last day of the experiment (day 21).

Thickening of epidermal cell walls and deposition of phenolic compounds in the wall from the internode were recorded using freehand sections (Yeung, 1998). Sections were stained with toluidine blue O (TBO) (O'Brien and McCully, 1981) and phloroglucinol-HCl (phHCl) method (Jensen, 1962) to observe the thickening of epidermal cell walls and the deposition of phenolic compounds. The presence of polyphenols, especially lignin, in the cell walls was indicated by a blue green to green color when sections were stained with TBO and an orange to red color when sections are stained with phHCl.

Anatomical changes of epidermal and cortical cells in the internode of interest were determined using prepared sections (see next section) by following the changes of cell size (length and width of the treated plants over the period of 21 days).

Stem segments were fixed in 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05M phosphate buffer, pH 6.8, for 24h at 4°C. After fixation, the segments were dehydrated in methyl cellosolve (BDH Chemicals) for 24h, followed by two changes of 100% ethanol for 24h each at 4°C. They were infiltrated gradually (3:1. 1:1, and 1:3 100% ethanol: Historesin, 24 hours each) with Historesin (Leica Canada, Markham, Ontario), followed by two changes of pure Historesin. The stem segments were then embedded according to Yeung (1999). Longitudinal serial sections, 3 μm thick, were cut

using a Ralph knife on a Reichert-Jung 2040 Autocut rotary microtome. Sections were stained according to Yeung (1984) with Periodic Acid - Schiff's (PAS) reaction for total carbohydrates and counter-stained with either 0.05% (w/v) toluidine blue O (TBO) in benzoate buffer for general histology or 1% (w/v) amido black 10B in 7% acetic acid for protein (Yeung, 1984). The sections were viewed under a Leitz photomicroscope. The images were captured using a Leica DF480 digital camera and the length of cells was determined using a micrometer. Cells from the same internodes from at least 10 ramets and 20 epidermal and adjacent cortical cells were measured.

MT arrays of elongating cells of alpine and prairie ecotypes of S. longipes

The first visible internodes from the apex of both ecotypes were collected at different time points of the growth period (day 0, 5, 8, 11 and 14). Immunofluorescent staining for examination of the orientation of MTs was carried out according to the method of Balŭska et al. (1992) using Steedman's wax (Steedman, 1957). Paraffin wax, the classical embedding medium of histology and anatomy, is unsuitable here because its relatively high melting-point destroys MTs and renders many other proteins non-immunoreactive. Steedman's wax, however, has a melting point of 35-37°C, is soluble in ethanol, has sectioning properties very similar to paraffin wax and has proved suitable for the immunofluorescence detection of microtubules (Baluška et al., 1992).

Internodes of interest were collected and fixed immediately with freshly prepared 4% paraformaldehyde in the MT stabilizing buffer (MTSB - 50mM PIPES, 5mM $MgSO_4$ and 50 mM EGTA, pH 6.9) for 1h at 20°C and overnight at 4°C. Following a brief rinse in MTSB, the samples were dehydrated in a graded ethanol series. The Steedman's wax

for embedding tissue was prepared from polyethylene glycol 400 distearate and 1-hexadecanol mixed in proportions 9:1 (w/w) (Balŭska et al., 1992). Internode segments were infiltrated at room temperature with mixtures of absolute ethanol plus wax made up in the proportions 2:1, 1:1 and 1:2 (v/v) at each step, followed by three changes of pure wax to remove the last traces of ethanol from the tissues. The infiltrated segments were then embedded by allowing the wax to solidify at room temperature. Sections were cut at a thickness of 10 μm and mounted on Superfrost Plus® slides (Fisher Scientific). Sections were then allowed to expand on a drop of distilled water and adhere to the slides. In order to facilitate penetration of the antibodies, the sections were first dewaxed in ethanol, rehydrated in an ethanol series, and allowed to stand in PBS for 20 min. The sections were then incubated in a blocking solution (1% bovine serum albumin and 1% goat serum, v/v) for 20 min. After draining of excess blocking solution, the sections were incubated with a mouse monoclonal antibody (anti-α-tubulin clone B5-1-2 from Sigma Aldrich diluted 1:150 in blocking solution overnight. After three washes in PBS solution (10 min each), they were stained with isothiocyanate-(FITC-) conjugated anti-mouse IgG raised in goat (Sigma Aldrich), diluted 1:200 in blocking solution for 2h at room temperature. After rinsing in PBS solution, the slides were mounted using an antifadant mountant (Citiflor). The prepared slides were then examined using a photomicroscope equipped with epifluorescence and standard FITC exciter and barrier filters. The images were captured using a Leica 500 digital camera.

Data analysis

Data on stem and internode elongation in two ecotypes were analysed by means of a three-way repeated measures analysis of variance (ANOVA). Honestly significant

difference (HSD) Tukey test was used to determine differences between treatments (SAS Institute Inc., 2004). The data are reported as mean ± standard error of the mean and differences are considered significant at $P < 0.05$.

3.3 Results

Stem and internode elongation of alpine and prairie ecotypes of S. longipes grown under two contrasting temperature regimes

It was shown in Chapter 2 that significant differences in stem length were observed between the alpine and prairie ecotypes grown under different temperatures. Ecotype and temperature significantly interacted to regulate stem growth (Table 1). The alpine and prairie ecotypes had a different magnitude of response to the same temperature treatment. The cumulative shoot growth of the prairie ecotype was significantly retarded when plants were grown under cooler temperature regime (10°/4°C) (Fig. 2.1A) than under a warmer temperature regime (28°/22°C) (Fig. 1B). The alpine ecotype also exhibited significant stem elongation in response to warm temperatures (Fig. 2.1B) and its growth was retarded when plants were grown under cooler temperatures (Fig. 2.1A).

Growth of the internode of both alpine and prairie ecotypes was similar to that of the stem elongation (Fig. 2.4A, B). Warmer temperature regimes promoted internodal elongation. Plants grown under cooler temperatures maintained a short stem with short internodes (Fig. 2.4A). The internodes of the prairie ecotype elongated better than the alpine under both conditions (Fig. 2.4A, B). The elongation of internodes followed an acropetal growth pattern (the centre of growth is shifted towards the shoot apex).

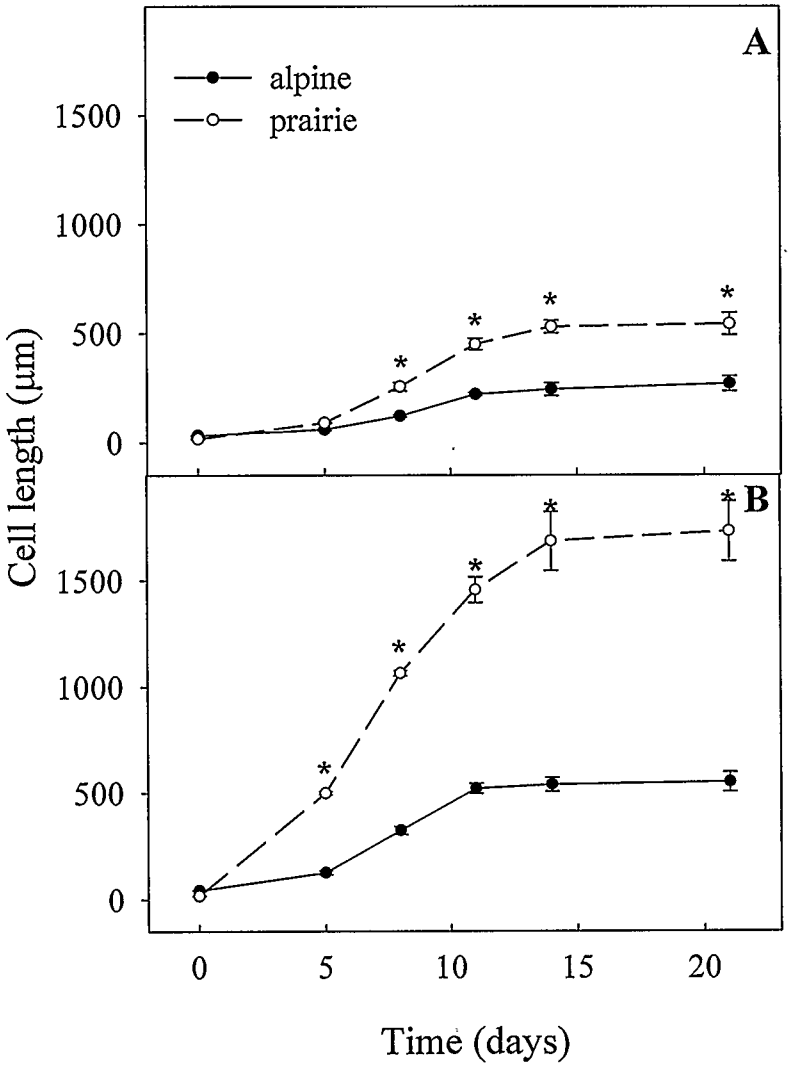

Figure 3.1. Epidermal cell elongation of alpine (closed circle) and prairie (open circle) ecotypes of *Stellaria longipes* grown under (A) cooler (10°/4°C, day and night) and (B) warmer (28°/22°C, day and night) temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean. Symbols that are surmounted by an asterisk indicate significant difference between the two ecotypes at each time point according to Tukey's multiple comparison test ($P < 0.05$)

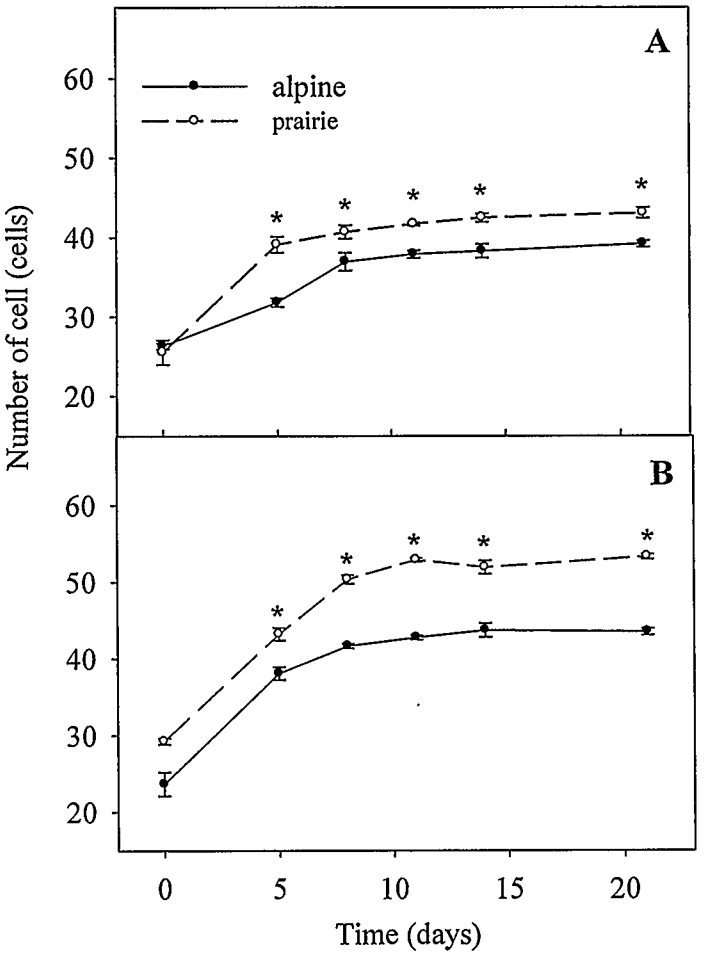

Figure 3.2. Linear cell number of alpine (closed circle) and prairie (open circle) ecotypes of *Stellaria longipes* grown under (A) cooler (10°/4°C, day and night) and (B) warmer (28°/22°C, day and night) temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean. Symbols that are surmounted by an asterisk indicate significant difference between the two ecotypes at each time point according to Tukey's multiple comparison test ($P < 0.05$)

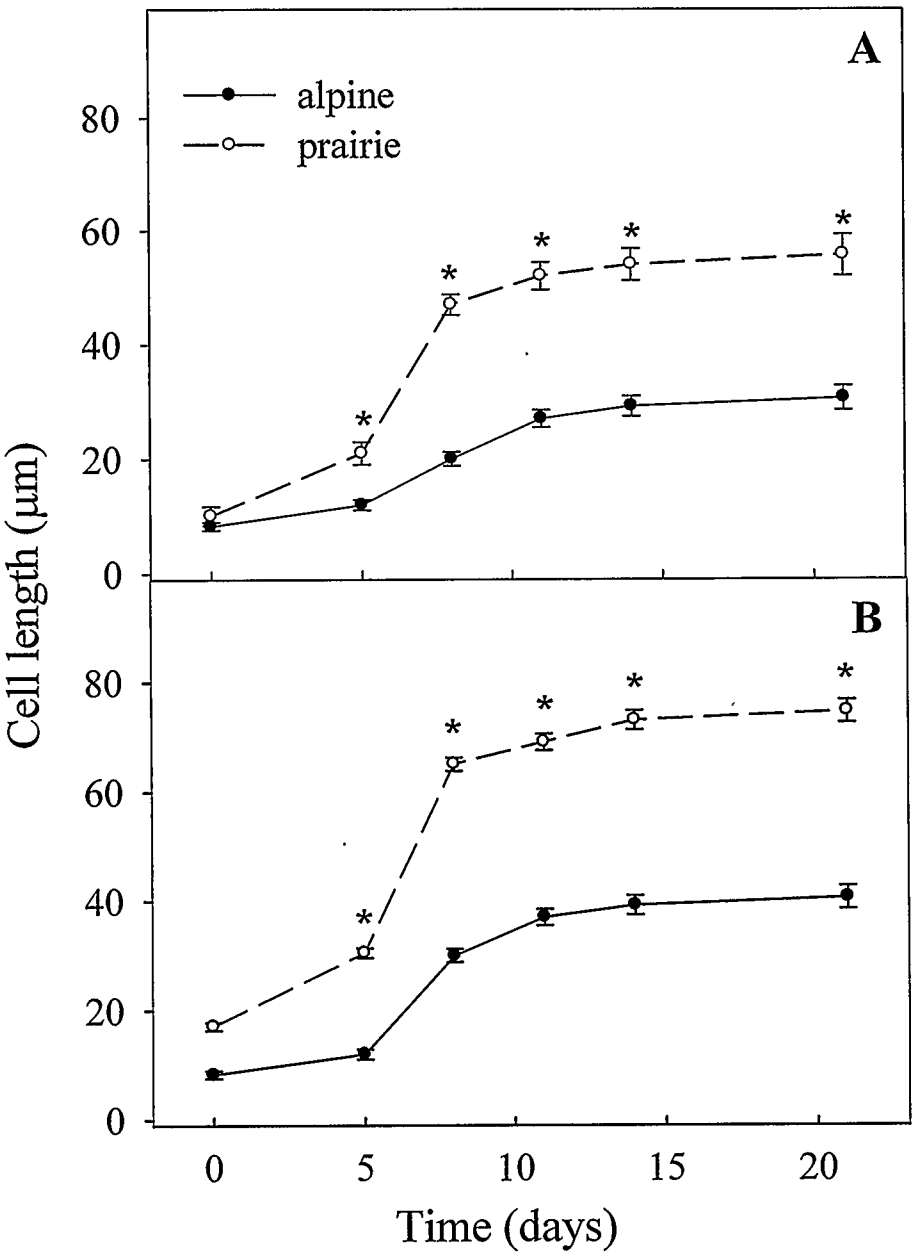
A
B
alpine
prairie
Cell length (µm)
Time (days)
0
20
40
60
80
0
5
10
15
20
*

Figure 3.3. Cortical cell elongation of alpine (closed circle) and prairie (open circle) ecotypes of *Stellaria longipes* grown under (A) cooler (10°/4°C, day and night) and (B) warmer (28°/22°C, day and night) temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean. Symbols that are surmounted by an asterisk indicate significant difference between the two ecotypes at each time point according to Tukey's multiple comparison test ($P < 0.05$)

Table 3.1. Repeated measures ANOVA (F value) of the effect of temperature on stem and internode elongation of alpine and prairie ecotypes of *Stellaria longipes* from day 0 to day 21. Significance levels: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$

Source	Stem elongation	Internode elongation
Ecotype	20979.20***	499.32
Temperature	15270.70***	664.00***
Ecotype x temperature	7687.48***	115.09
Ecotype x time	2278.94***	155.08***
Time x temperature	1231.75***	216.02***
Ecotype x time x temperature	787.17***	36.84***

Differences in cellular morphology of alpine and prairie stem segments in response to contrasting temperature regimes

Measurements of epidermal and cortical cells showed that epidermal cells expanded rapidly upon transfer to different temperature regimes. Cells of the prairie ecotype elongated more than the alpine. Cells of plants grown under cooler temperature regime elongated slowly and the elongation phase did not last for more than 5 days (Fig. 3.1A). Prolonged warm conditions promoted the axial elongation of epidermal cells, resulting in an approximately 100 times increase in length in the prairie ecotype cells and 50 times increase in the alpine ecotype (Fig. 3.1B).

The number of axial epidermal cells in the first internode also increased during the course of treatment (Fig. 3.2). There was a significant difference in the number of cells between the alpine and prairie ecotypes. The prairie ecotype always had more cells per internode than the alpine. Unlike the rapid elongation activity in epidermal cells, cortical cells elongated to a lesser extent. Cortical cell length increased at the first few days and then remained constant (Fig. 3.3). Changes in temperatures resulted in a significant increase in elongation and division of epidermal and cortical cells of the prairie ecotype. By contrast, there was little increase in cell number as well as cell length in the alpine ecotype grown under the same conditions (Figs. 3.2 and 3.3).

Data on cell width of plants grown in warm conditions showed that cell width of both ecotypes first increased rapidly by day five, followed by a decrease in width by 21 (Fig. 3.4B). In cooler conditions, there was an opposite trend of expansion of cells in the alpine and prairie ecotypes. Cell width of alpine ecotype decreased overtime. However,

cell width of the prairie increased for the first few days and decreased toward the end (Fig. 3.4A).

Closer examination of the cross sections of alpine and prairie stem segments grown under different temperature treatments revealed histological differences between both ecotypes. The most notable difference could be found in the epidermal cell walls. Epidermal cell walls thickened faster when plants were grown under cooler temperatures (Fig. 3.5). The cell walls became gradually thicker as time progressed. The walls of the alpine were thicker than those of the prairie (Fig. 3.5C, D, G, H). Positive staining was noted with the ph-HCl stain. The red staining pattern of epidermal walls of both ecotypes grown under cold temperatures was observed on day five (Fig. 3.5A, C), indicating that phenolic compounds (lignin) were deposited earlier than in plants grown in warmer condition (Fig. 3.5B, D). Also, stem sections from the alpine ecotype stained darker with phHCl than those from the prairie ecotype, suggesting a larger amount of phenolic compounds present in its cell walls. The phenolic compounds were deposited much earlier in the alpine ecotype than in the prairie ecotype (Fig. 3.5). Judging from the staining intensity, there was also a differential level of phenolic compounds accumulating in the cells of plants grown in cooler, compared to the warmer temperature regime. The TBO stainpattern also confirmed the presence of phenolic compounds (lignin) in the epidermal walls. Initially, the cell walls stained purple at the time of transfer to different temperature regimes (data not shown). As the plant ceased to elongate, the cell walls gradually thickened and the blue coloration intensified (data not shown).

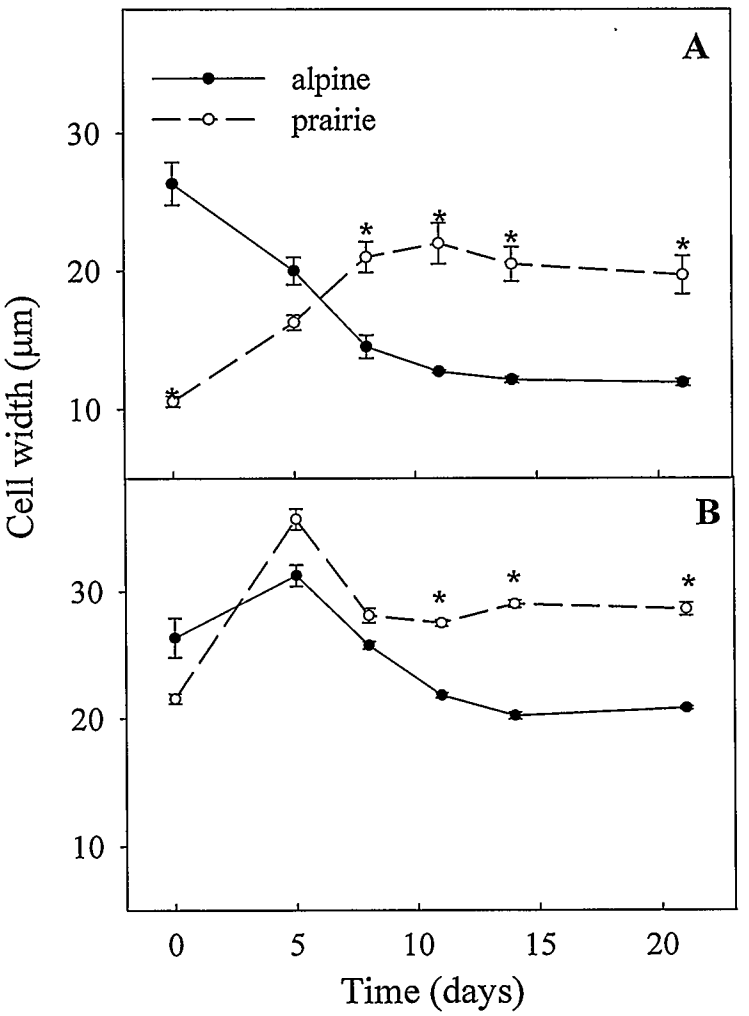

Figure 3.4. Epidermal cell width of alpine (closed circle) and prairie (open circle) ecotypes of *Stellaria longipes* grown under (A) cooler (10°/4°C, day and night) and (B) warmer (28°/22°C, day and night) temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean. Symbols that are surmounted by an asterisk indicate significant difference between the two ecotypes at each time point according to Tukey's multiple comparison test ($P < 0.05$)

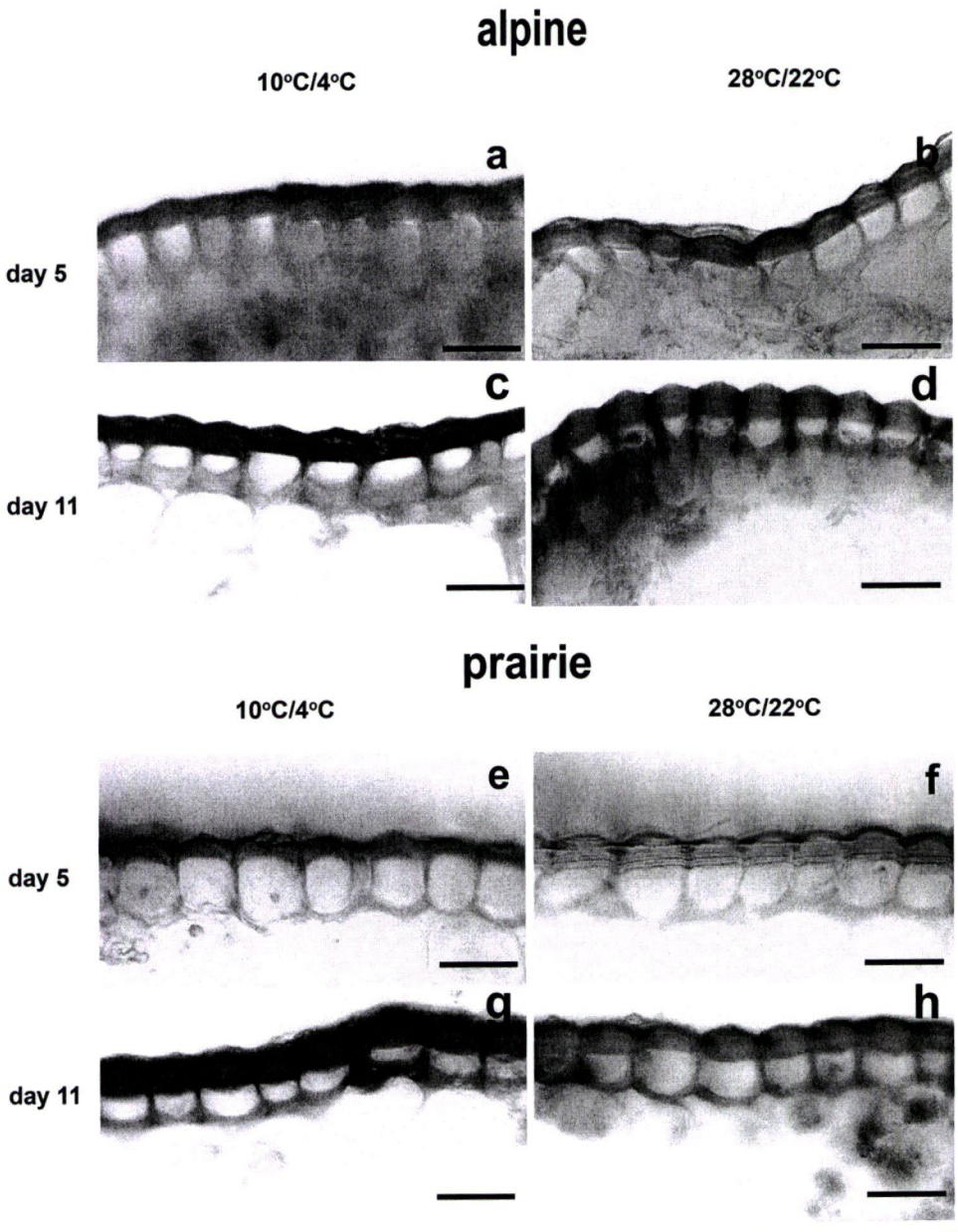

Figure 3.5. Micrographs of epidermal cells in cross sections of the alpine (A, B, C and D) and prairie (E, F, G and H) ecotypes of *Stellaria longipes* grown under cooler ($10^{\circ}/4^{\circ}C$, day and night) and warmer ($28^{\circ}/22^{\circ}C$, day and night) temperature regimes from day 5 to day 11. The red staining pattern of epidermal wall of both ecotypes indicated that there were more phenolic compound that were deposited to alpine than in prairie ecotype. The thickness of the cell wall indicated how much cell wall material was deposited to the cells. Bars: 25 μm.

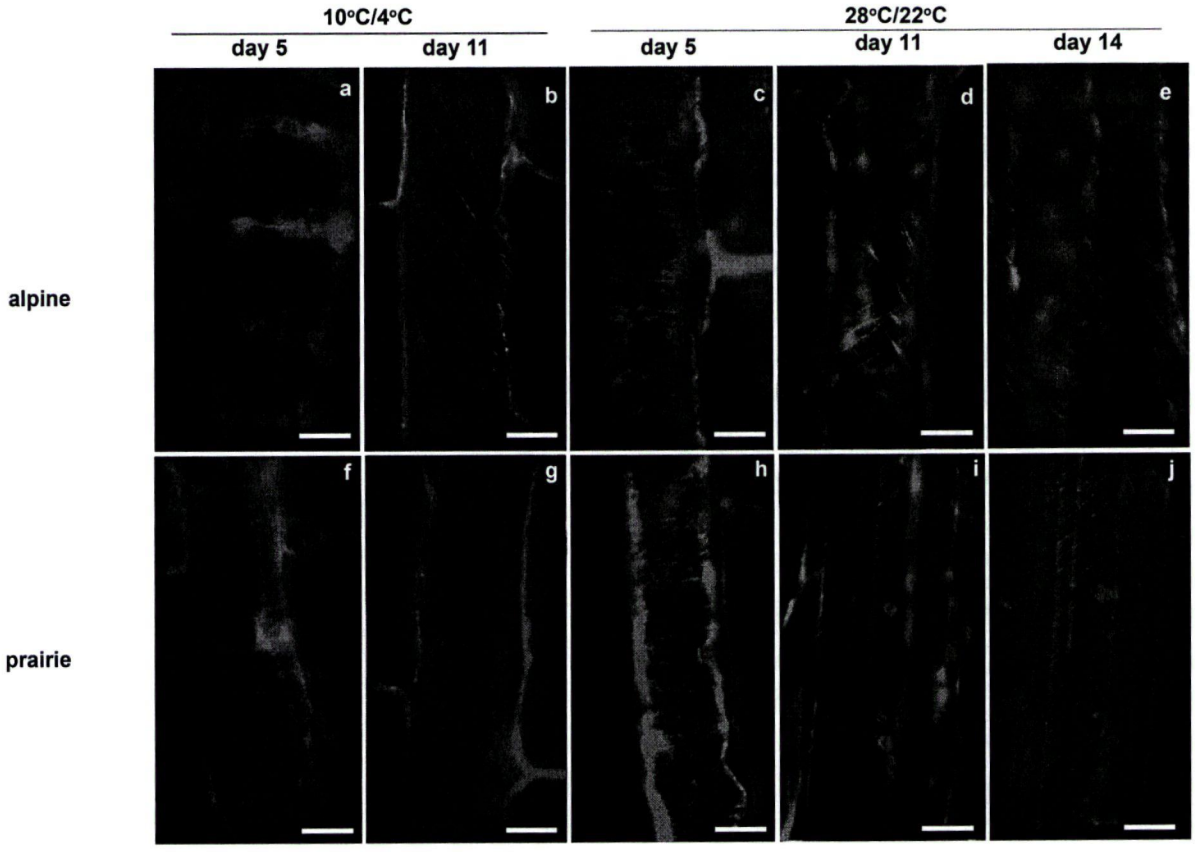

Figure 3.6. Micrographs of immunolabelled microtubules of cells from alpine (A to E) and prairie ecotypes (F to H) of *Stellaria longipes* grown under cooler (10°/4°C, day and night) and warmer (28°/22°C, day and night) temperature regimes from day 5 to day 14. Microtubules (MTs) were transversely orientated when plants were grown under both conditions by day 5 (A, F, C and H). By day 11, MTs reoriented obliquely (B, J, D and I). MTs were found criss-crossing by day 14 (E and J). Cells of alpine ecotype showed more criss-crossing MTs (B and E) than those of the prairie ecotype (G and J) do not. Bars: 25 μm.

Orientation of cortical MTs in the alpine and prairie ecotypes

Longitudinal sections of the first visible internodes were used to observe changes of the cortical MTs during the course of elongation. The most notable difference observed in the distribution of MTs was that more MTs were observed when plants were maintained at warmer temperatures (28°/22°C) when compared to the cooler temperatures (10°/4°C) (Fig. 3.6). During the rapid elongation phase (day 5), transversely oriented MTs were observed in both ecotypes at both temperatures. Thereafter, the elongation process slowed down and that was when MTs reoriented from a transverse to an oblique and criss-crossing direction (Fig. 3.6B, G, D, E, I and J). As elongation slowed, MTs became less abundant in the cytoplasm (Fig. 3.6)

For those plants maintained at warmer temperatures, a similar change in the orientation was found (Figs. 3.6D and I); however, MTs remained abundant within the cytoplasm. For the alpine ecotype, elongation ceased around day 11 (Fig. 3). Coinciding with the cessation of growth, MTs gradually became sparse (Fig. 3.6D and E). On the other hand, the prairie ecotype continued to elongate till day 14; MTs maintained an oblique to a criss-crossing pattern (Fig. 3.6J) and were more abundant within the cytoplasm (Fig. 3.6I and J).

3.4 Discussion

Differences in cellular structures potentially explain the resulting responses of alpine and prairie ecotypes

The primary objectives of this research was to determine the effect of two contrasting temperature regimes on stem elongation of alpine and prairie ecotypes and to explain differences in the responses of these ecotypes using a histological approach.

Different temperature regimes significantly regulated the stem elongation of both ecotypes. Warmer temperatures promoted stem elongation whereas cooler conditions retarded it, suggesting that temperature plays a significant role in the response. The difference in the elongation ability of these two ecotypes provides an ideal comparative system to study the subcellular structures that underlie their stem elongation responses.

In order to study the effects of temperature on stem growth, the first visible internode from the terminal shoot bud was chosen because internodes that were formed before the inductive signal elongate first and, therefore, are responsible for the initial phases of the increase in stem growth (Sachs, 1965). The attained internodal length was shown to be a result of the increase in cell number and cell length. The most obvious morphological change in the expanding internodes following temperature treatments was an increase in the number of cells (Fig. 3.2). Results from this study is in accordance with the study of Kende et al. (1998) who reported that rapid internodal elongation was a result of an increased cell division activity in the intercalary meristem and an enhanced elongation of the newly formed cells of deepwater rice. Metzger and Dusbabek (1991) also observed an increase in the number of cells in pennycress following thermoinduction. Studies on the cellular anatomy of fully grown internodes of Easter lily have also shown that temperature affects their growth through changes in cell length, or both cell length and the number of cells (Erwin et al., 1994). Results of our experiment indicate that increased cell division and elongation in response to warmer temperatures were factors that directly contributed to internodal growth, hence, stem elongation. Moreover, differences in cell elongation and cell division processes regulated the difference in the elongation ability of these two ecotypes. Increase in temperatures

resulted in extensive cell division and cell elongation in rapidly elongating zone of the stems of the prairie ecotype as compared to the alpine. Alpine plants continuously produced new leaves but the internodes elongated very slowly because of the low cell division and the cell elongation rate. Although increased cell division following temperature treatment cannot contribute directly to growth, cell division does provide more cells that elongate more or less to the same extent as those in cold-grown plants during early stage of rapid elongation (Jones, 1983; Metraux, 1987; Metzger and Dusbakek, 1991). Therefore, the primary cellular process that controls temperature-induced stem elongation in *S. longipes* is cell division, although it can only potentiate the maximum length the stem will ultimately attain. Heupel and Kutschera (1996; 1997) also demonstrated that cell reproduction is a precondition for stem elongation and that both cell reproduction and cell elongation greatly contribute to the growth of the sunflower hypocotyls.

Histochemical staining of phenolic compounds provides insights into the stem elongation in *S. longipes*. The thickening of the cell walls and the deposition of phenolic compounds can play important roles in stem elongation (Kutschera, 2000). During cell expansion, new polymers must be incorporated into the growing wall otherwise it would thin until ruptured by turgor pressure (Richmon et al., 1980). This implies that wall biosynthesis and cell expansion are coordinately regulated and interdependent. However, many studies showed that cell wall thickness is reduced (Sargent et al., 1974; Schnepf and Deichgraber, 1979; Kutschera, 1990; Refregier et al., 2004), increased (Fujino and Itoh, 1998), or unchanged (Ray, 1962). Recently, Derbyshire et al. (2007) suggested that wall biosynthesis varies depending upon the cell type and the stage of growth. In *S.*

longipes, it is shown that cell walls of both ecotypes grown under cold conditions thickened faster and phenolic compounds were deposited earlier than plants grown under warm conditions. Therefore, low temperatures induced thickening of peripheral walls and an early deposition of phenolic compounds to the stem cells, which resulted in the stiffening of cell walls, therefore, reduction in wall plasticity and elongation retardation of the internodes. These processes were less retarded under warmer conditions, resulting in rapid expansion of the cells. Interestingly, the thickening of the cell walls and deposition of phenolic compounds occurred earlier in the alpine ecotype, therefore resulted in a less elongation plasticity for it. Chuong et al. (2001) reported that completion of endodermis formation and lignification of epidermal walls in the stems of a prairie genotype of *S. longipes* occurred after internodal cells had ceased to elongate. Iiyama et al. (1994) also recommended that covalent cross-linking between cell wall polymers significantly terminated the phase of wall extension. Kutschera (2000) suggested that thickening of the mature cell walls during elongation time causes stiffening of the tissues and organ in the plant body. My experiment showed that wall thickness and deposition of phenolic compounds to the wall during rapid elongation stage of alpine and prairie ecotypes is exceptionally dynamic, is dependent on ecotypes, and is under tight control of growth conditions.

The other important parameter that should be taken into consideration when explaining the difference in the ability of alpine and prairie cells to elongate was the cell width. The cell width increased at the beginning of the treatment when the cells were dividing which may enable the cells to accommodate subsequent stretching of cells. The ability to increase cell width at the onset of axial elongation contributes to the differences

observed between the two ecotypes. The epidermal cells in the prairie plants increased in width during the first few days of elongation at both temperatures whereas in the alpine ecotype only increase in cell width was observed at warmer temperatures (Fig. 3.4). This added width may better accommodate the axial elongation and allow axial growth. The subsequent decrease in cell width coincided with the time of rapid increase in cell length (Fig. 3.3). This clearly indicates that the cells were being stretched. This observation indicates that the prairie cells could elongate better because they were more plastic in accommodating axial elongation in response to temperature treatments. Cells of alpine ecotype did not have that ability possibly because the cell wall material and phenolic compounds were deposited in the cells earlier, preventing them from stretching thereby causing the cessation of cell elongation.

One other factor that may also control cell wall extensibility is the cytoskeleton, especially microtubules (MTs) (Cosgrove, 2005). In this study, warmer temperatures resulted in a more abundance of MTs within the cytoplasm of both ecotypes. This clearly indicates one of the positive functions of warmer temperatures in plant growth is by mediating the abundance of MTs within cells. The orientation of cortical MTs in the cells is considered as a prerequisite for the rapid expansion of the cell that occurs in the elongation zone (Nemoto et al., 2004). Transversely aligned microtubules have been considered to promote cells to expand longitudinally (Erhard and Shaw, 2006). The difference in the dynamics of MTs in alpine and prairie ecotypes could explain the difference in their cell elongation under the same condition. Having MTs reoriented in the oblique criss-crossing pattern earlier the cell elongation process slowed down in the alpine ecotype, whereas that of the prairie ecotype continued on for a few more days.

Therefore, the dynamics of MTs distribution significantly contributed to the potential difference in the cell elongation and eventually stem elongation of these ecotypes.

In conclusion, temperature significantly affects stem elongation of alpine and prairie ecotypes of *S. longipes*. Obvious retardation of growth was documented in plants grown in cooler temperatures, whereas growth was considerably enhanced when plants were grown under warmer conditions. The profound difference in the responses of both ecotypes to different temperature regimes makes these ecotypes a good system to explore the key genetic and physiological differences that underlie the responses. Overall, difference in the timing of cell wall thickening, deposition of lignin and cross-linking of cell wall material as well as the dynamics of cortical MTs might have caused difference in the elongation, division and stretching ability of the cells. These may be some of the important factors that regulate the differences in the stem elongation plasticity of alpine and prairie ecotypes of *S. longipes*.

Chapter 4

Possible role of ethylene in the stem elongation of alpine and prairie ecotypes of *Stellaria longipes* grown under different temperature regimes

4.1 Introduction

Ethylene, a gaseous plant hormone, is well-known for its major role in inhibition of plant stem elongation (Abeles et al., 1992). The effect of ethylene on stem elongation has also been controversial. In darkness, ethylene inhibits elongation, but under some conditions in the light, ethylene promotes elongation (Smallet et al., 1997). While a large number of studies indicate that exogenous ethylene inhibits stem elongation, ethylene has also been shown to promote stem elongation in some plant species. For instance, a low concentration of applied ethylene can increase the stem elongation in tobacco under shade (Peirik et al., 2003). Also, low concentration of ethylene was reported to promote leaf cell expansion in sunflower (Lee and Reid, 1997). However, it was suggested that a stimulation of elongation growth by ethylene may occur particularly at relatively low concentration (Fiorani et al., 2002). In deep water rice (Kende et al., 1998), other semi-aquatic plants (Jackson, 1985) and *Stellaria longipes* (Emery, 1994; Emery et al., 1994), ethylene is essential for internodal and stem elongation. Although the mechanism of ethylene action on stem elongation still remains unclear, it has been implicated in determining the orientation of microtubules and microfibrils of cytoskeleton (Abeles et al., 1992). Evidence indicates that ethylene influences the cell elongation by changing the orientation of microtubules and microfibrils of the cytoskeleton (Steen and Chadwick, 1981; Le et al., 2005).

In *S. longipes*, upon exposure to wind stress in controlled conditions, the alpine ecotype, but not the prairie ecotype, showed a significant increase in ethylene evolution and a reduction in growth (Emery et al., 1994). Kurepin et al. (2006a) reported that ethylene was possibly a controlling factor for stem growth, especially in the alpine ecotype. Reductions in ethylene evolution resulted in increased stem elongation of alpine *S. longipes* and vice versa (Kurepin et al., 2006a).

Many studies have been carried out to understand the effect of environmental factors, especially light, on ethylene evolution. Recently, it was reported that increased ethylene evolution in the alpine ecotype of *S. longipes* during rapid stem elongation and extended photoperiods plays a growth regulatory role in this ecotype (Walton et al., 2006). However, not much work has been done to elucidate the effect of temperature on the ethylene evolution and how it correlates to the stem elongation of plants grown under different temperature regimes. It was shown, from previous experiment, that temperature exerted different effects on the growth of alpine and prairie plants. Variation in the elongation of ecotypes under the same conditions is most likely due to differences in their ability to respond to specific temperature regimes. In this chapter, I examined the effects of changing temperature regimes on ethylene evolution by the alpine and prairie ecotypes of *S. longipes* in an effort to further understand the role of this key plant growth regulator in plants adapted to different environmental conditions.

4.2 Materials and methods

Plant material

Plant material was prepared as described in Chapter 2.

Growth chambers with varied temperature conditions

In order to study the ethylene evolution of alpine and prairie ecotypes to varied temperature regimes, a minimum of four growth chambers are required. The conditions of these chambers are set as follow:

Treatment	Temperature (°C)		
	(8:00 am – 4:00 pm)	(4:00 pm – 12:00 pm)	(12:00 pm – 8:00 am)
1	10	10	4
2	16	16	10
3	22	22	16
4	28	28	22

The light irradiance and Red/Far red (R/FR) light ratio was maintained as ~120 μmol m^{-2} s^{-1} and 1.76 ± 0.02, respectively.

Evolution of ethylene from tissues

Individual ramets were excised from the stem at the soil/air interface and harvested shoots were placed in a 3-ml syringes (BD syringes) with a three-way valve and sealed with a plunger held at the 1.5 ml mark. After a 15 min incubation (at room temperature, ~ 45 μmol m^{-2} s^{-1}), 1ml of gas were transferred to a second gas tight syringe through the three-way valve (note that wound induced ethylene was not detected for a least 45 min – Emery, 1994). This gas sample was analyzed for ethylene using a Photovac 10Splus gas chromatograph (Photovac Inc., Markham, Ontario) equipped with a photoionization detector and a 40/60 Carbopack B column (Supelco, Canada, Oakville, Ontario).

Ethephon and STS treatment

Ethephon was prepared on the day of use by diluting an appropriate amount in pH 3 distilled water. A series of ethephon concentrations (0, 0.01, 0.1 M) were applied by brushing it on to each ecotype such that each pot received a different concentration. Control plants were applied with pH 3 distilled water to match the acidic pH of the ethephon solution. Increments in growth were recorded on day 0, 5, 8, 11, 14 and 21.

Silver thiosulphate (STS) was prepared on the day of use from stock solutions of 0.01M $AgNO_3$ and 0.04 M $Na_2S_2O_3$ stored in the dark. Equal volumes of these chemicals were thoroughly mixed produce 2.5 mM STS. Then it was diluted to reach the concentrations of 0.01 and 0.02 M. Fresh STS was applied by brushing onto plants.

The response to applied STS and ethephon and temperature of detached segments of alpine and prairie ecotypes of S. longipes

- Plant material

Three pots of each ecotype were selected for one treatment. A minimum of 50 segments of each ecotype were detached from the plants grown under assigned temperature regimes 5 days after transfer from LDW and gathered in a 500ml beaker. A sterilizing mixture, which included 100ml of distilled water, 50ml of bleach, and 1 to 2 drops of tween 20, was added to each beaker. After being sterilized for at least 10 min, a 3mm segment was detached from the first internode below the shoot apex and cultured in assigned hormone/nutrient media.

- Culture media

In this experiment, 1/4 MS medium (Murashige and Skoog, 1962) supplemented 0.5% sucrose was used. The pH was adjusted to 5.8 prior to autoclaving at 121°C, 1 atm for 30 minutes. Ethephon (at concentrations of 0.1, 0.01, 0.001M) and STS (at concentrations of 0.01, 0.001M) were sterilized and added to medium after autoclaving as optional additives according to the experimental purposes.

Media were then pipetted into 24-well culture plates at a volume of 250µl per well before detached segments were placed in. The tissue culture plates had been sealed with paraffin to avoid contamination before the tissue culture plates were placed under controlled temperature regimes mentioned above.

4.3 Results

Evolution of ethylene from plants grown under different temperature regimes

Ethylene was actively produced by the alpine and prairie ecotypes during the 21 day growth period. However, the levels of ethylene evolution were different among temperature treatments and between the two ecotypes (Fig. 4.1).

As for the alpine ecotype, the starting ethylene level was 1.5 times higher than that of the prairie ecotype. There were differences in ethylene evolution among temperature treatments. Ethylene evolution was highest when plants were grown under 28°/22°C and lowest when plants were grown under 16°/10°C. Ethylene produced by the plants grown under 10°/4°C and 22°/16°C were not significantly different. The ethylene levels fluctuated over time. Ethylene levels picked up on day 11. After this point, ethylene levels started declining again and stayed somewhat stable until day 21 (Fig. 4.1A).

Ethylene evolution of the prairie ecotype was different. The starting ethylene level was significantly lower than that of the alpine ecotype. Ethylene levels from plants grown under 22°/16°C and 28°/22°C stayed somewhat stable until day 11, then dropped very quickly by day 14 and increased remarkably afterwards. Plants that were grown under cooler temperatures (10°/4°C and 16°/10°C) did not follow the same trend. Starting from day 0, ethylene levels slowly increased until day 8 where it went up on day 11 and went down until it stayed stable by day 14. There was no significant difference in ethylene levels on day 11 among temperature treatments as compared to other time points (Fig. 4.1B).

Stem elongation of the alpine and prairie ecotypes treated with ethephon and STS

Ethephon and STS had opposite effects when they were applied to the alpine ecotype (Fig. 4.2). Application of STS to the alpine plants grown under 10°/4°C resulted in higher stem length as compared to control plants. Application of ethephon at concentration of 0.01 M did not significantly affect stem elongation of the alpine plants, but 0.1 M significantly inhibited growth of the alpine plants grown under this condition (Fig. 4.2A). STS continued to have positive effect on stem growth when it was applied at concentration of 0.01 M to the alpine plants grown under 16°/10°C. Higher concentration of STS (0.02M) inhibited growth of the alpine plants although the effect was not significant until day 14 of the growth period. The application of ethephon at all concentrations inhibited growth of the alpine plants grown under this condition (Fig. 4.2B). Similar effects of ethephon and STS were seen when the alpine plants were grown

under warmer temperature regimes (Fig. 4.2 C, D). STS at concentrations of 0.01 M promoted stem elongation but STS at higher concentration (0.02 M) inhibited it. Ethephon at all concentrations negatively affected the stem elongation of the alpine plants grown under these conditions (Fig. 4.2 C, D).

Ethephon and STS also had different effects when applied to the prairie ecotype (Fig. 4.3). Under 10°/4°C, ethephon at all concentrations strongly inhibited stem elongation of the prairie ecotype. STS at concentration of 10^{-2}M significantly promoted growth whereas STS at concentration of 0.02 M had no effect (Fig. 4.3A). Similar effects were observed when plants were grown under 16°/10°C (Fig. 4.3B). Application of STS and ethephon inhibited growth of plants grown under warmer temperature conditions (Fig. 4.3C). Regardless of the concentrations of STS that was applied, they all inhibited the growth of the prairie plants. The inhibitory effects caused by ethephon were more profound than that of STS (Fig. 4.3C). The stem elongation of plants grown under 28°/22°C was neither inhibited nor promoted when they were applied with different concentration of STS. There was no significant difference among control plants and plants applied with STS. Ethephon significantly inhibited the stem elongation of the prairie plants grown under this condition.

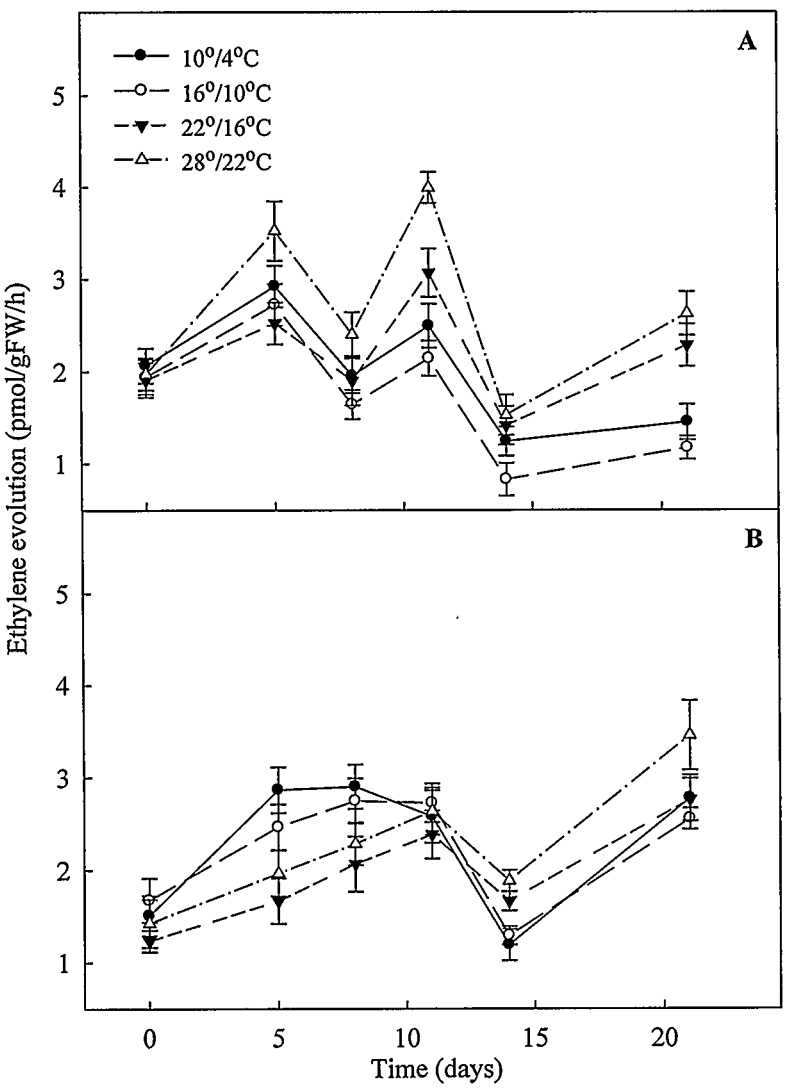

Figure 4.1. Ethylene evolution of alpine (A) and prairie (B) ecotypes grown under four different temperature regimes from day 0 to day 21. The error bars indicate standard error of the mean.

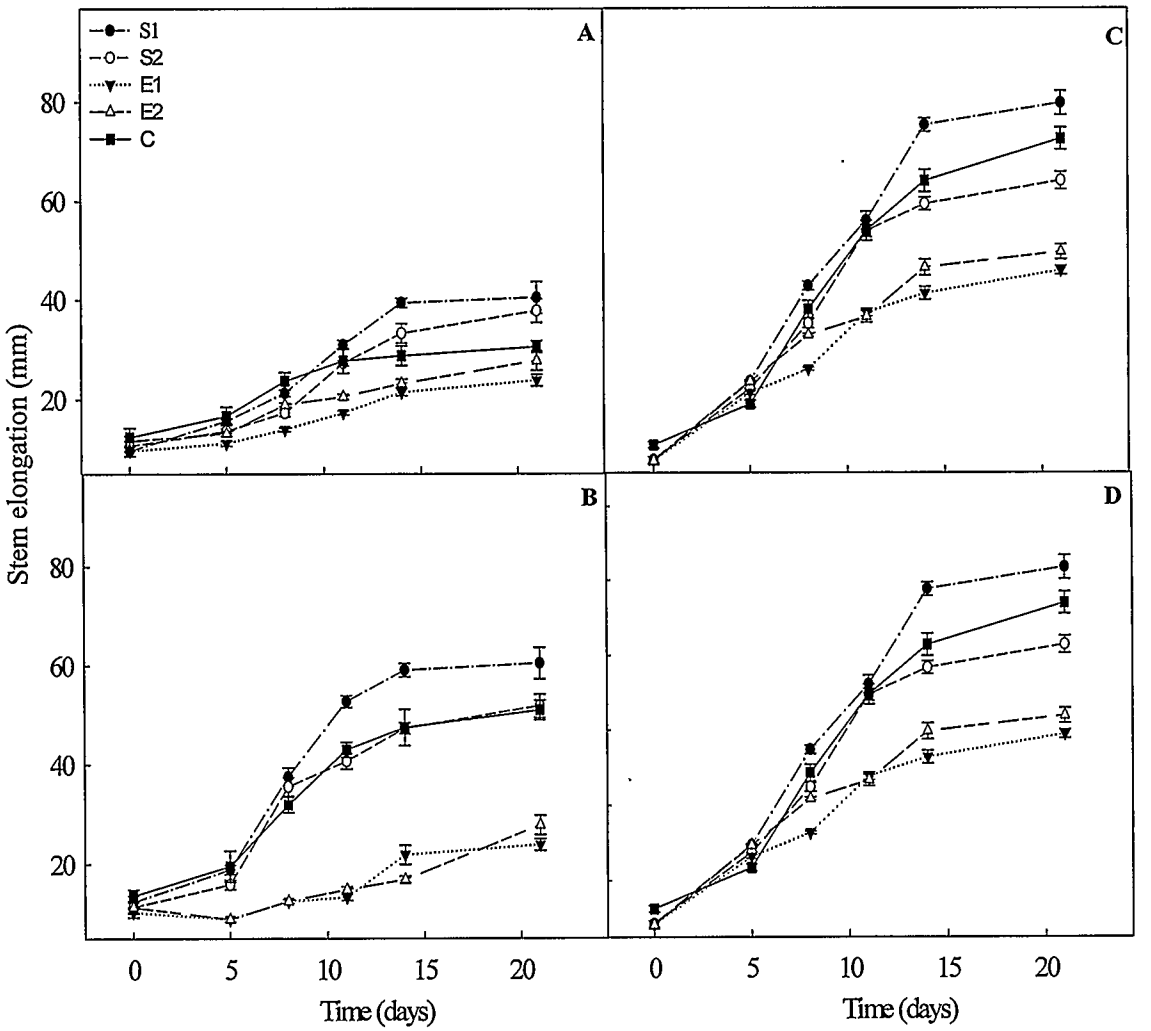

Figure 4.2. Stem elongation of alpine ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations of ethephon and STS. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
E1:	Ethephon at concentration of 0.1M
E2:	Ethephon at concentration of 0.01M
S1:	STS at concentration of 0.01 M
S2:	STS at concentration of 0.02 M

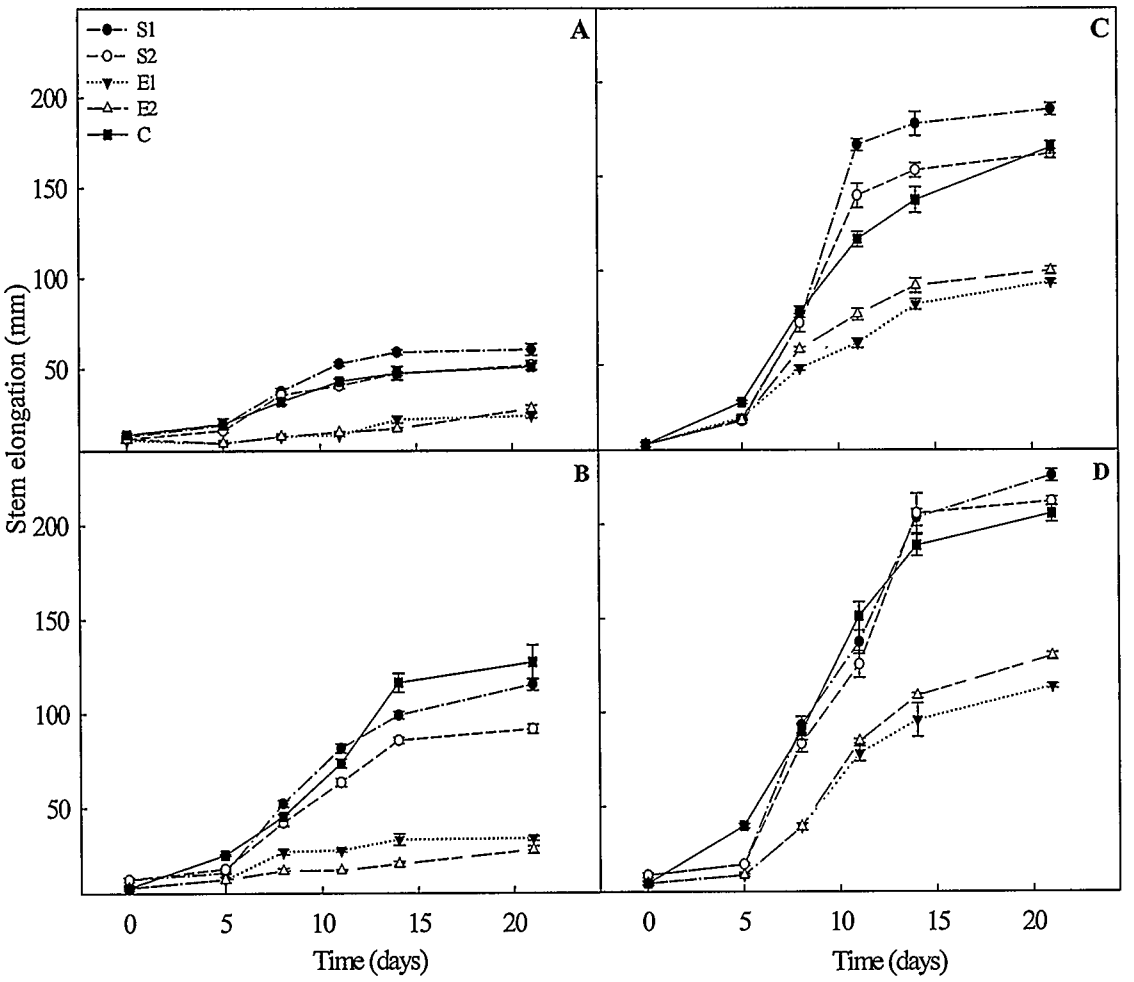

Figure 4.3. Stem elongation of prairie ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations of ethephon and STS. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
E1:	Ethephon at concentration of 0.1M
E2:	Ethephon at concentration of 0.01M
S1:	STS at concentration of 0.01 M
S2:	STS at concentration of 0.02 M

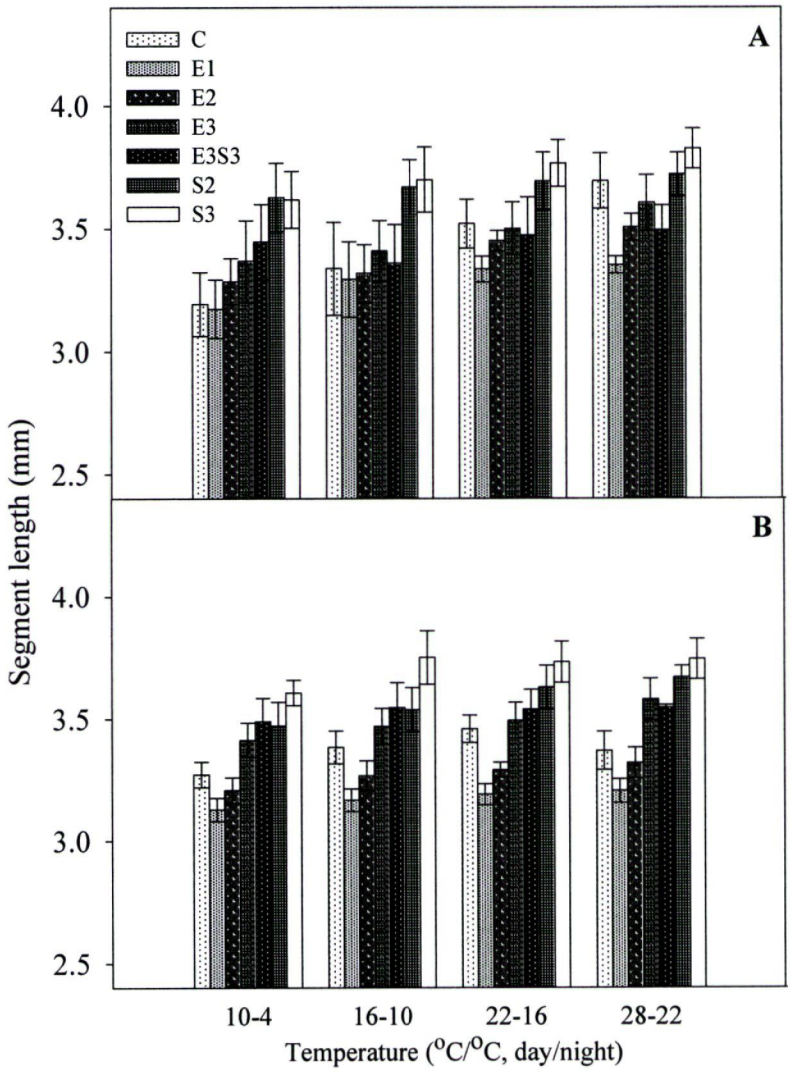

Figure 4.4. Segment elongation of alpine (A) and prairie (B) ecotypes of *Stellaria longipes* grown under four different temperature regimes treated with different concentrations of ethephon and STS. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
E1:	Ethephon at concentration of 0.1M
E2:	Ethephon at concentration of 0.01M
E3:	Ethephon at concentration of 0.001M
S1:	STS at concentration of 0.01 M
S2:	STS at concentration of 0.001 M
E3S3:	Ethephon at concentration of 0.001M in combination with STS at concentration of 0.001M

Application of ethephon and STS did not have significant effects on the elongation of the alpine segments when they were cultured under 10°/4°C, 16°/10°C and 22°/16°C (Fig. 4.4A). Neither inhibitory nor promoting effects were seen under these conditions regardless of concentrations of chemicals applied. When segments were cultured under the warmest temperature regime (28°/22°C), application of both STS and ethephon resulted in shorter segments as compared to the control. Ethephon had a stronger inhibitory effect than STS (Fig. 4.4A). As for the prairie ecotypes, application of ethephon inhibited the elongation of segments cultured under all spectrum of temperature. STS, however, had different effects when segments were cultured under different temperature regimes. For instance, application of STS promoted growth of segments grown under 10°/4°C and 16°/10°C but it did not have any effects when applied to segments grown under warmer temperature regimes (22°/16°C, 28°/22°C) (Fig. 4.4B).

4.4 Discussion

Prolonged warm temperature induced differing stem elongation responses in the alpine and prairie ecotypes of *S. longipes*. The prairie ecotype consistently exhibited greater stem elongation than the alpine ecotype (Chapter 2). They were more responsive to changes in temperature regimes. Although the dwarf appearance of the alpine plants did not remain with increasing temperature conditions, this ecotype still maintained shorter stems than the prairie ecotype. The optimum temperature for ethylene production is near 30°C. The rate of ethylene production declines above 30°C until production ceases near 40°C. Production of stress ethylene has been observed when plants are exposed to damaging temperatures. Damage and stress ethylene have been reported when plants are

cooled below critical chilling temperatures or when they are heated above 40°C. However, these temperatures might change depending on plant species (Abeles et al., 1992). If the temperature treatments are sublethal, and tissue repair occurs, ethylene production returns to normal (Abeles et al., 1992). In this experiment, it was hypothesized that plants which underwent too cold or too warm temperature regimes would produce more ethylene, which would then inhibit the stem elongation of plants grown under these conditions. Difference in the magnitude of elongation between alpine and prairie ecotypes might be partially due to the difference in the level of ethylene evolution by these ecotypes.

The present experiments indicate that ethylene might be a significant factor that accounts for the difference in the stem elongation of alpine and prairie ecotypes in varied temperature regimes. The alpine ecotype processed different ethylene production level to prairie plants. Inhibition of ramet elongation was positively correlated with increases in ethephon concentration. In addition, elongation increased after treatment with STS (Fig. 4.3 A, B). The same response has been well documented in crop species (Lieberman, 1979; Reid, 1988; Abeles et al., 1992) as well as wild species (Emery et al., 1994; Kurepin et al., 2006; Walton et al., 2006). It has been suggested that the major role of ethylene may be restricted to signaling environmental or physiological alteration (Klee et al., 1991). Upon exposure to hot temperature regimes (28°/22°C), the alpine ecotype showed a marked increase in ethylene production but not an increase in growth. Prolonged exposure to heat may cause a stress on plants, resulting in higher levels of ethylene. A similar effect was not observed on plants under 22°/16°C possibly because that is the best condition for plant growth. Therefore, stress-inducing ethylene evolution

should not be recorded. Exposure of the alpine plants to other conditions did not result in remarkable changes in ethylene levels, although plants generated more ethylene when they were placed under cold (10°/4°C and 16°/10°C) temperature regimes.

Investigation of the prairie ecotype revealed a different effect of ethylene in controlling plant growth. Upon exposure to cold, ethylene production increased remarkably, resulting in shorter stems. Ethylene is a well-known inhibitor of stem elongation (Abeles et al., 1992) and my result is consistent with this statement. Lower ethylene levels recorded when plants were grown under warm conditions further support our initial hypothesis. However, it does not agree with other studies that suggested ethylene promotes growth of the prairie ecotype (Emery et al., 1994). Ethylene can be considered as an inhibitory agent in stem elongation of prairie ecotype if the response of these plants to ethephon, STS and temperature are considered together. Firstly, application of ethephon inhibited growth. Secondly, treatment with STS at concentration of 0.01 M promoted growth. Thirdly, ethylene production was changed in accordance to changes in temperature regimes. However, if ethylene was a promoting factor to stem elongation, then an increase in elongation would have been expected during day 14 and day 21 of the experiment. It is possible that a different inhibitory process was overriding ethylene effects at this point of time. In fact, one cannot explain the difference in response of these two ecotypes to temperature based solely on difference in ethylene levels at certain times. Dynamics of other growth regulators, such as auxin or gibberellins might involved in this process. Results from my study showed that the effects of warm temperature are similar to extended photoperiod or shade avoidance response of *S. longipes* (Kurepin et al., 2006; Walton et al., 2006). However, both alpine and prairie

ecotypes of *S. longipes* showed reduction in stem lengths when exposed to high concentration of ethylene (Miranda et al., 2001).

While exogenous ethylene strongly influenced stem elongation of the intact alpine and prairie plants, it did, of course, have a pronounced effect on detached segments. The ethylene effect on excised stem sections and intact plants indicated that ethylene inhibits stem elongation in whole green plants either by inhibiting basipetal IAA translocation (Burg and Burg, 1966), by affecting IAA metabolism in some manner (Morgan and Gausman, 1966), or by some other auxin-independent action.

Although the mechanism of ethylene action on stem elongation still remains unclear, ethylene has been implicated in determining the orientation of microtubules and microfibrils of cytoskeleton. The normal orientation of microtubules is along the circumference inside the plasma membrane and vertical to the plane of cell division (Abeles et al., 1992). Evidence indicates that ethylene influences cell elongation by changing the orientation of microtubules and microfibrils of the cytoskeleton (Steen and Chadwick,1981). It is possible that the difference in cellular structure and dynamics in MTs that were observed in Chapter 3 were partially regulated by ethylene. However, more work needs to be done to confirm this.

Differences in response to temperature between alpine and prairie ecotypes, especially when temperature is too cold or too warm may be due to differences in dynamics of ethylene evolution. Since the prairie ecotype is stimulated to grow under warm temperature regimes, and thus having less ethylene than its counterpart, this maybe an important factor that is missing in the alpine ecotype. The alpine ecotype maintained the ability to produce ethylene under cold conditions to inhibit grow and prevent

exceeded stem elongation which is fatal in a cold and windy environment. At the same time, the prairie ecotype appeared to evolve a mechanism, which resulted from a specialization gear toward optimizing growth in a warmer but competitive environment. Further research on the endogenous levels of other phytohormones, such as gibberellins and auxin, and their interaction with ethylene would provide more insight into the hormonal regulation of *S. longipes* in response to different temperature treatments.

Chapter 5

Possible roles of GA_3 and IAA on stem elongation of the alpine and prairie ecotypes of *Stellaria longipes* grown under different temperature regimes

5.1 Introduction

Plant growth and development are regulated by both external environmental factors, such as light and temperature, and by a set of endogenous regulators collectively known as the plant growth regulators (PGRs). PGRs mediate many of responses that facilitate adaptation to environmental changes. Auxin, brassinosteroids, and gibberellins (GAs) promote growth, whereas cytokinins and abscisic acid (ABA) have growth inhibitory effects (Koonneef and Karssen, 1994; Clouse, 1996).

The appropriate degree of stem elongation is vital for a plant's survival in a competitive environment. Stem elongation of tomato (*Lycopersicon esculentum*) in response to temperature was supposed to result from an alteration in the carbohydrate status in the elongating region of the stem (Went and Bonner, 1943). However, study on the temperature-induced stem elongation of lily (*Lilium longiflorum*) suggested that total carbohydrate availability within the plant was not the primary factor responsible for the stem elongation response to temperature but differences in hormone synthesis or action are (Erwin et al., 1989).

Gibberellins (GAs) are involved in many aspects of plant development, especially stem elongation. Current hypotheses to explain the control of stem elongation include changes in gibberellin levels (Ross et al., 1992), gibberellin sensitivity (Reid et al., 1990), auxin levels (Law and Davies, 1990), and levels of a hypothetical growth inhibitor

(Bruinsma and Hasegawa, 1990). It is clear that not all growth-regulating factors act via the same mechanisms. For instance, in peas, the response to light may be controlled via changes in GA sensitivity (Reid, 1988), while the ontogeny appeared to involve changes in GA_1 levels (Ross et al, 1992). Exogenous applied bioactive GAs reduce or eliminate the inhibition of stem elongation caused by unfavorable temperature regimes (Myster and Moe, 1995; Grindal et al., 1998a). GA levels were reported to involve in temperature effects on growth of pea (Tangeras, 1979; Ross et al., 1989; Grindal et al., 1998a) and Italian bellflower (*Campanula isophylla*) (Moe, 1990). Alternatively, other PGR, such as IAA, might be involved in this process. It has been suggested that IAA, in addition to GAs, is an essential factor for stem elongation, and that these two hormones may control separate processes that together contribute to stem elongation (Yang et al., 1996). Gray et al. (1998) demonstrated that high temperature could promote the elongation of the hypocotyl in light-grown *A. thaliana* seedlings, and that this growth response is partly depended on IAA. Furthermore, it was found that the observed thermoperiodic response on stem elongation in *A. thaliana* could be mediated through changes in the levels of IAA (Thingnaes et al., 2003).

Despite being the object of a large number of studies, the physiological background of temperature-induced stem elongation remains an open question. Many descriptive models have been developed for stem elongation in chrysanthemum (Khattak and Pearson, 1997; Schouten et al., 2002), pea (Yang et al., 1993; 1996), and Arabidopsis (Gray et al., 1998; Thingnaes et al., 2003). However, these models lack insight into the physiological basis of stem elongation process of wild species, such as *Stellaria longipes*. The results from Chapter 2 indicated that temperature has strong effect on stem

elongation of alpine and prairie ecotypes of *S. longipes*. The aim of this study was to indirectly uncover the response and the possible role of gibberelic acid (GA_3) and auxin (IAA) in stem elongation of alpine and prairie ecotypes of *S. longipes* grown under different temperature regimes by exogenously applying plant growth regulators on intact plants and detached segments. It was hypothesised that differences in response and sensitivity to IAA and GA_3 partly explain differences in stem elongation of the alpine and prairie ecotypes grown under different temperature regimes.

5.2 Materials and methods

Plant material

Plant material was prepared as described in Chapter 2.

Growth chambers with varied temperature conditions

Growth chambers and temperature regimes were set as described in Chapter 4.

Experimental design

Effects of applied plant growth regulators (PGRs) on stem elongation of the alpine and prairie ecotypes grown under different temperature regimes

- Application of GA_3 and AMO 1618

Three pots of each ecotype were selected for each treatment.

GA_3 solutions were made up in 5 ml 95% aqueous ethanol and diluted with distilled water to the desired concentration. GA_3 was applied as 10 ml spraying solution to the whole plant from different directions. The same amount of ethanol was applied to the control ramets. For each ecotype, GA_3 was applied to a minimum of 20 ramets at the following doses: 0, 10^{-3}, 10^{-4}, 10^{-5} M on day 5, 11 and 14 of their growth period.

AMO 1618 was used as GAs biosynthesis inhibitor. AMO 1618 solutions were dissolved in 5 ml of 95% aqueous ethanol and diluted with distilled water to the desired concentrations. AMO 1618 was applied as 10 ml spraying solution to the whole plant from different directions. Ramets were also sprayed with ethanol as control. AMO 1618 was applied to at least 20 ramets at the following doses: 0, 10^{-2}, 10^{-3}M on day 5, 11 and 14 of their growth period.

In a separate treatment, AMO 1618 at concentration of 10^{-4}M was applied to ramets which also received 10^{-4}M of GA_3 in order to reverse any inhibitor-induced growth inhibition.

- Application of IAA and TIBA

Three pots of each ecotype were selected for each treatment.

IAA solutions were made up in 5 ml 95% aqueous ethanol and diluted slowly with distilled water (pH 8) to the desired concentration. IAA was applied as 10 ml spraying solution to the whole plant from different directions. The same amount of ethanol was applied to the control ramets. For each ecotype, IAA was applied to a minimum of 20 ramets at the following doses: 0, 10^{-4}, 10^{-5}, 10^{-6} M on day 5, 11 and 14 of the growth period.

TIBA was used as auxin transport inhibitor. TIBA solutions were dissolved in 5 ml of 95% aqueous ethanol and diluted with warm distilled water to the desired concentrations. TIBA was applied as 10 ml spraying solution to the whole plant from different directions. Ramets were also sprayed with ethanol as control. TIBA was applied to at least 20 ramets at each of doses: 0, 10^{-3}, 10^{-4}, 10^{-5} M on day 5, 11 and 14 of the growth period.

To confirm whether the effects of TIBA are due to their inhibition of auxin and not a side effect, a control treatment in which TIBA (10^{-4}M) and auxin (10^{-4}M) were added together was carried out. In the control treatment, the effect of the inhibitor should be overcome resulting in normal growth.

Response to PGRs of detached segments of alpine and prairie ecotypes of S. longipes grown under different temperature regimes

Three pots of each ecotype were selected for each treatment. A minimum of 50 segments of each ecotype were detached from the plants grown under four temperature regimes on day 5 and gathered in a 500-ml-beaker. A sterilizing mixture, which included 100 ml of distilled water, 50 ml of bleach, and 1 to 2 drops of tween 20, was added to each beaker. After being sterilized for at least 10 min, a 3mm segment was detached from the first internode below the apex and cultured in assigned hormone/nutrient media.

- Culture media

For all experiments 1/4 MS medium (Murashige and Skoog, 1962) supplemented 0.5% sucrose was used. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C, 1 atm for 30 minutes. Plant growth regulators, including GA_3, IAA, AMO 1618, TIBA were sterilized and added to medium after autoclaving as optional additives according to the experimental purposes.

Media was pipetted into 24-well culture plates at a volume of 250µl per well before detached segments were placed in it. The tissue culture plates were sealed with paraffin to avoid contamination before the tissue culture plates were placed under controlled temperature regimes mentioned in Chapter 4.

- PGRs

IAA, GA_3 and their correspondent inhibitors, TIBA and AMO 1618 respectively, were prepared for this experiment. All plant growth regulators (PGRs) were made up at concentrations of 10^{-3}, 10^{-4}, 10^{-5}M. PGRs were added separately to the wells. Solution of 1/4 MS medium was used as control.

Data analysis

Data were analysed by means of a three-way repeated measures analysis of variance (ANOVA). Honestly significant difference (HSD) Tukey test was used to determine differences between temperature treatments (SAS Institute Inc., 2004). The data are reported as mean ± standard error of the mean and differences are considered significant at $P < 0.05$.

5.3 Results

Effects of exogenous plant growth regulators on elongation of intact stems of alpine and prairie ecotypes of S. longipes.

Application of GA_3 and AMO 1618 had significant effects on stem elongation of the alpine ecotype grown under different temperature regimes. Although GA_3 and AMO were applied on day 5, the difference among treatments can only be observed starting from day 8 (Fig. 5.1). As temperature increased, the effects of GA_3 and AMO 1618 were more profound. Under 10°/4°C there was no significant difference among plants treated with AMO at concentrations of 10^{-3} and 10^{-4} M as well as plants treated with GA_3 at concentration of 10^{-5}M. Higher concentrations of GA_3 promoted elongation of plants

grown under this condition. The effects of exogenous GA_3 and AMO 1618 were more visible when plants were grown under warmer temperature regimes (Fig. 5.1 B, C, D). Under 16°/10°C, plants treated with GA_3 grew more compared to control plants. GA_3 at concentration of 10^{-4}M promoted growth better than GA_3 at concentration of 10^{-3}M. Under 22°/16°C, application of GA_3 on plants resulted in shorter stems. There was no difference in the stem elongation of plants treated with GA_3 and control plants, suggesting that saturated concentrations of GA_3 did not have positive effects on stem elongation of the alpine ecotype. Application of GA_3 at concentration of 10^{-5}M inhibited growth. Under 28°/22°C, however, application of GA_3 at all concentrations did not promote stem elongation of alpine plants to any extend. There was no significant difference in the stem elongation among control plants and plants treated with different concentrations of GA_3. Under all temperature regimes, AMO 1618 always inhibited growth of these plants, suggesting that GAs is very important in promoting growth of plants under these conditions.

Unlike the effects of exogenous GA_3 and AMO 1618 on alpine ecotype, GA_3 and AMO 1618 strongly affected stem elongation of the prairie ecotype. GA_3 at certain concentrations could promote plants growth whereas AMO 1618 significantly inhibited it (Fig. 5.2). Application of GA_3 somewhat promoted growth of plants grown under 10°/4°C although the effect was not significant. There was no difference in stem length of plants until day 14 although chemicals were applied from day 5 of the growth period (Fig. 5.2A). Under 16°/10°C, application of GA_3 at concentration of 10^{-4}M significantly promoted stem elongation of the prairie ecotype. Lower or higher concentrations of GA_3 did not result in longer stem as compared to the control plants (Fig. 5.2B). GA_3 and AMO 1618 had

stronger effects on the prairie plants when they were grown under warmer temperature regimes. Under 22°/16°C, application of GA_3 strongly promoted growth of the prairie ecotype and there was no difference among GA_3 treatments (Fig. 5.2C). Somewhat similar effects of exogenous GA_3 and AMO 1618 were seen on plants grown under 28°/22°C. There was no difference in stem elongation of plants treated with different concentrations of GA_3 and control plants. The effects of AMO 1618 were more profound when plants were grown under this temperature regime (Fig. 5.2D). Addition of GA_3 to AMO 1618 could rescued the stem elongation of both alpine and prairie ecotypes (data not shown).

Application of IAA and TIBA had different effects on plants (Fig. 5.3). Under 10°/4°C, applied IAA at 10^{-6} M significantly promoted stem elongation, whereas TIBA at concentrations of 10^{-3} M significantly inhibited that of the alpine plants. The effects were visible as early as day 8 (three days after the applications). The rest of the treatments neither promoted nor inhibited the stem elongation the alpine ecotype grown under this condition (Fig. 5.3 A). There were no significant effects of applied IAA or TIBA on plants grown under 16°/10°C. The alpine plants grown under these conditions did not show any difference in growth regardless of the applied concentrations of IAA or TIBA (Fig. 5.3B). The opposite effects were observed on the alpine plants grown under warmer conditions (Fig. 5.3C, D). Neither IAA nor TIBA promoted the stem elongation of the alpine plants grown under these conditions. The higher the applied concentrations, the more inhibitory effects were observed.

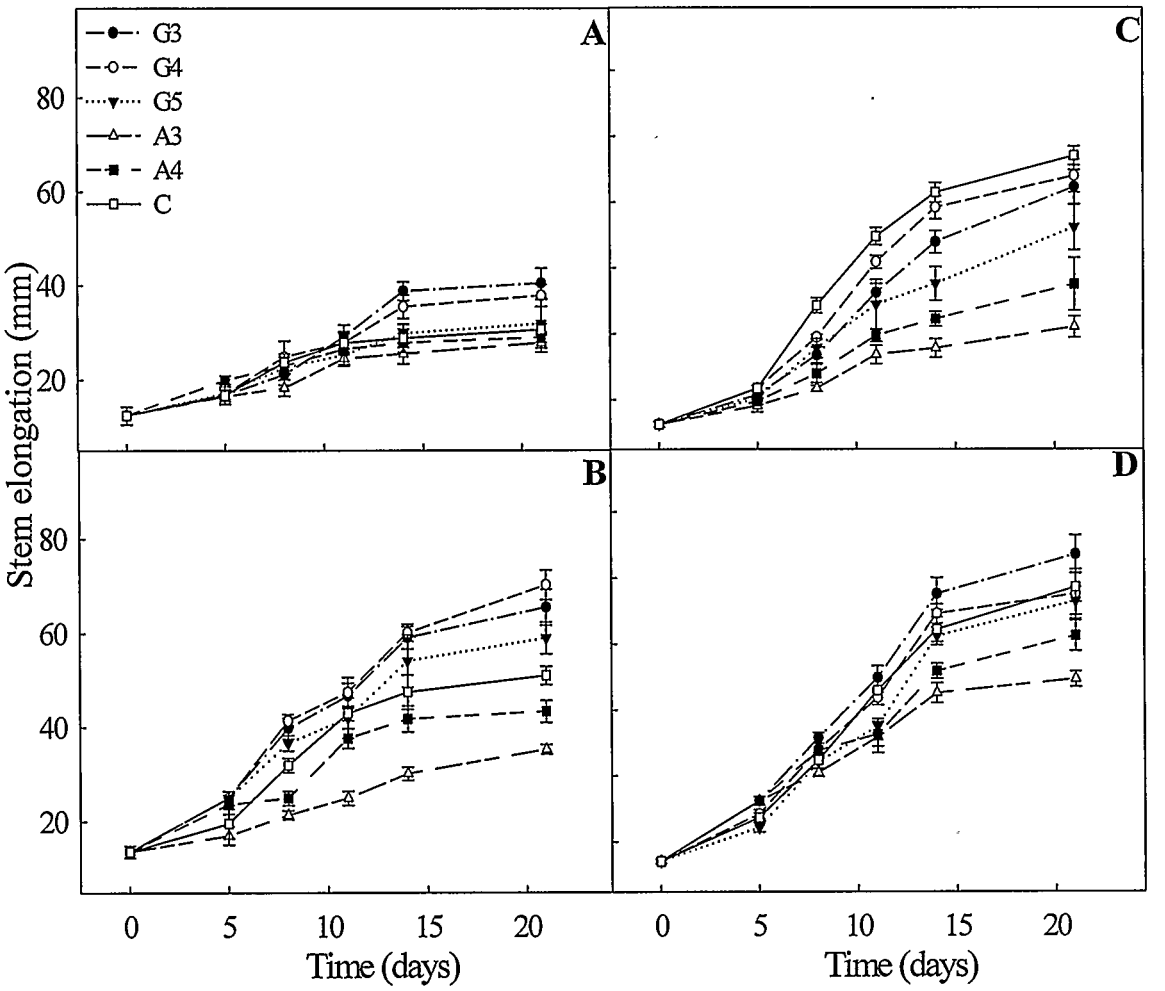

Figure 5.1. Stem elongation of alpine ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations GA_3 and AMO1618. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
G3:	GA_3 at concentration of 10^{-3}M
G4:	GA_3 at concentration of 10^{-4}M
G5:	GA_3 at concentration of 10^{-5}M
A3:	AMO 1618 at concentration of 10^{-3}M
A4:	AMO 1618 at concentration of 10^{-4}M

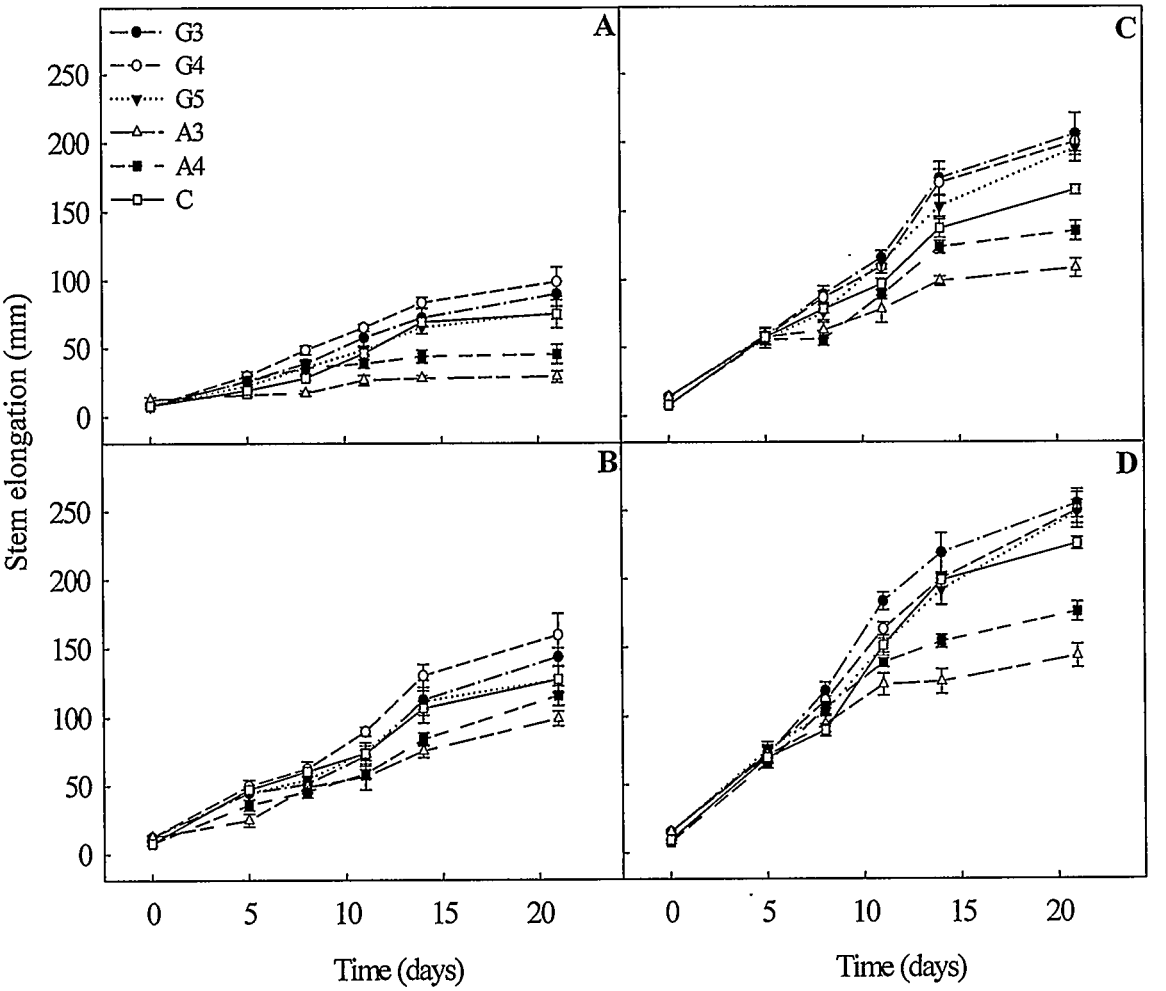

Figure 5.2. Stem elongation of prairie ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations of GA_3 and AMO1618. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
G3:	GA_3 at concentration of 10^{-3}M
G4:	GA_3 at concentration of 10^{-4}M
G5:	GA_3 at concentration of 10^{-5}M
A3:	AMO 1618 at concentration of 10^{-3}M
A4:	AMO 1618 at concentration of 10^{-4}M

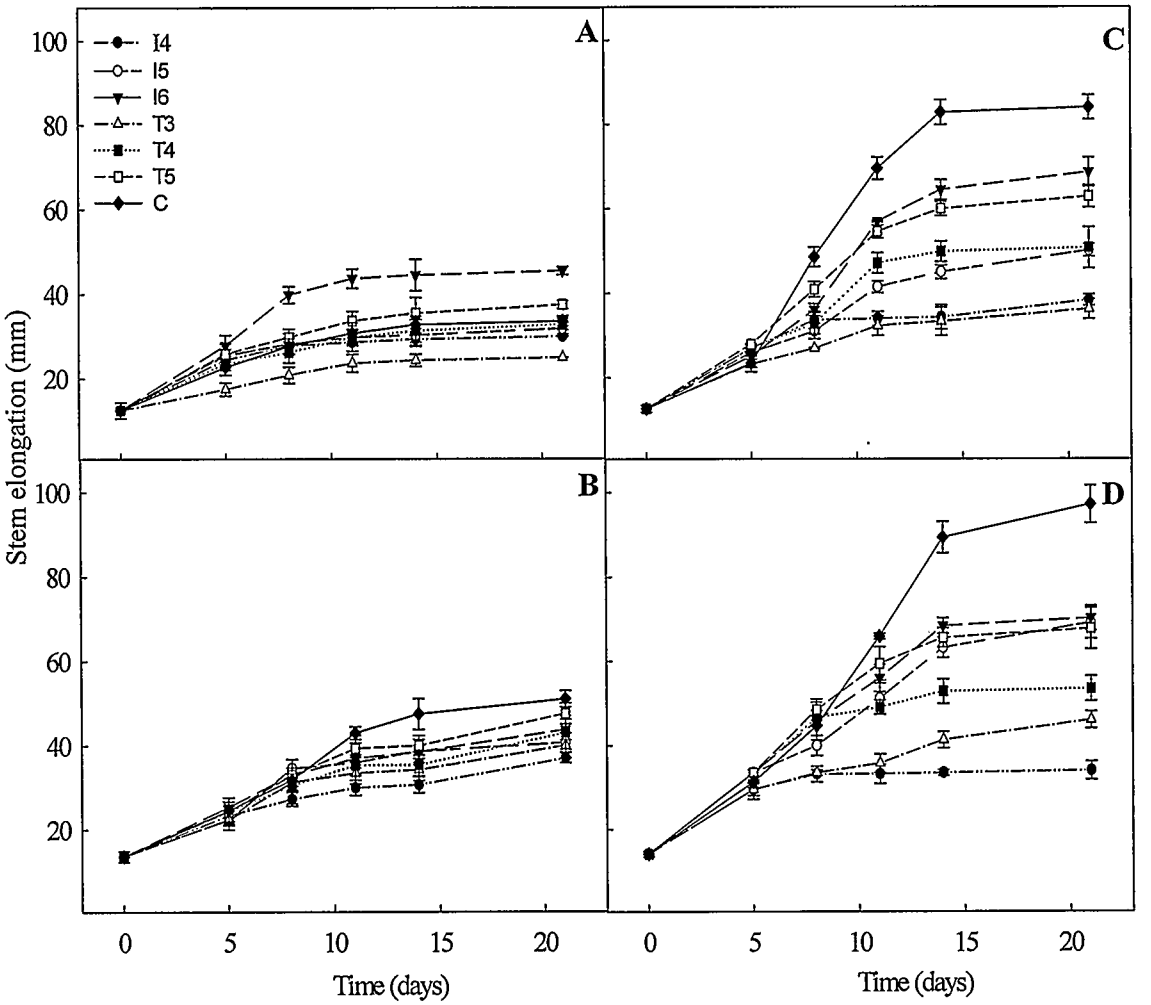

Figure 5.3. Stem elongation of alpine ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations IAA and TIBA.

Abbreviations:

C:	Control treatment
G3:	GA_3 at concentration of 10^{-3}M
G4:	GA_3 at concentration of 10^{-4}M
G5:	GA_3 at concentration of 10^{-5}M
A3:	AMO 1618 at concentration of 10^{-3}M
A4:	AMO 1618 at concentration of 10^{-4}M

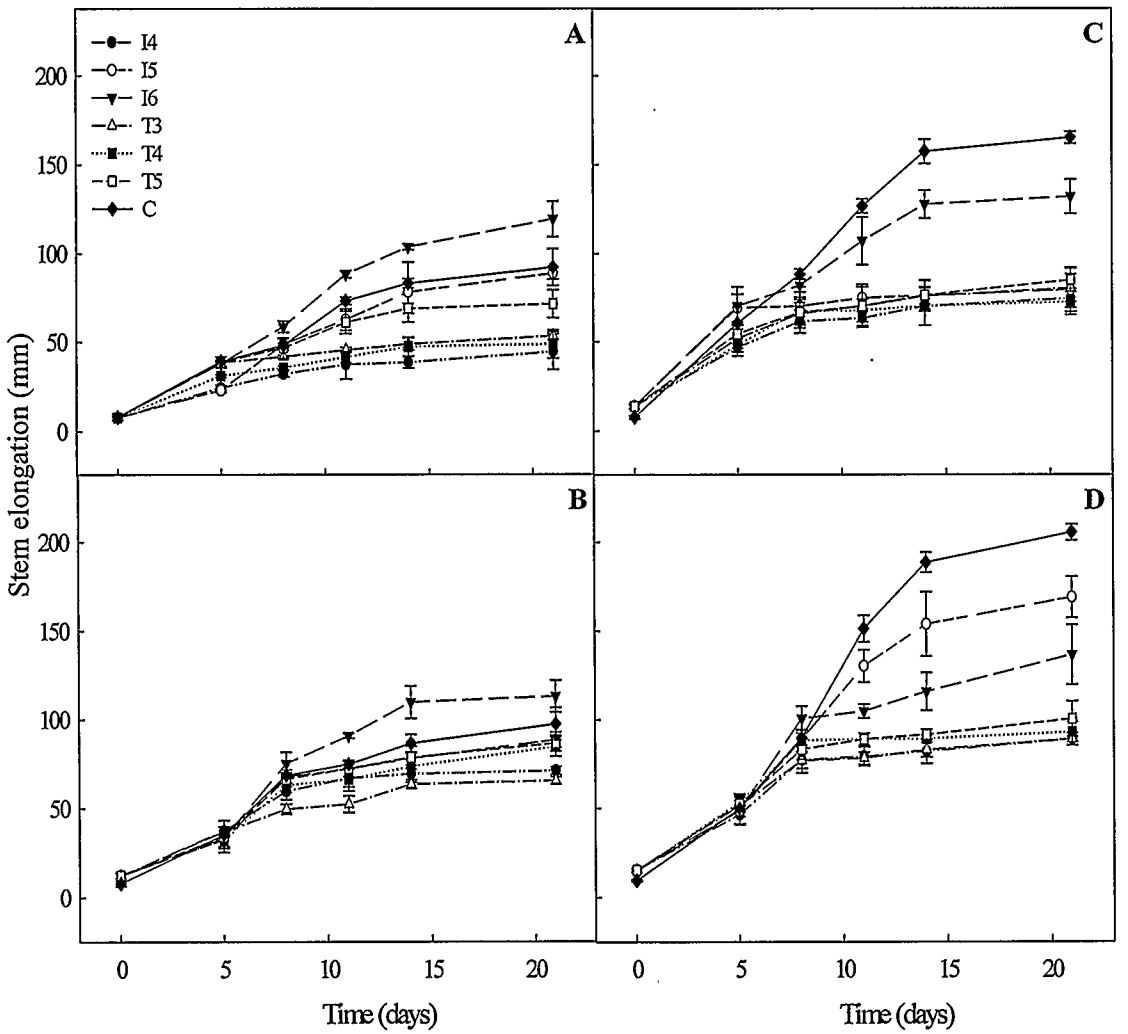

Figure 5.4. Stem elongation of prairie ecotype of *Stellaria longipes* grown under (A) 10°/4°C, (B) 16°/10°C, (C) 22°/16°C, (D) 28°/22°C (day/night) and treated with different concentrations IAA and TIBA. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
I4	IAA at concentration of 10^{-4}M
I5	IAA at concentration of 10^{-5}M
I6	IAA at concentration of 10^{-6}M
T3	TIBA at concentration of 10^{-3}M
T4	TIBA at concentration of 10^{-4}M
T5	TIBA at concentration of 10^{-5}M

Similar effects of IAA and TIBA were observed in the prairie ecotype (Fig. 5.4). Under 10°/4°C applications of IAA at concentration of 10^{-6} M significantly promoted stem elongation of the prairie plants. IAA at concentration of 10^{-5} M did not have any effects on the prairie plants. Plant growth was however significantly inhibited when treated with 10^{-3} M of IAA. IAA at this concentration actually had the same effects as TIBA, which inhibited stem elongation of the prairie ecotype (Fig. 5.4A). There was no difference in stem elongation of plants grown under 16°/10°C and treated with IAA at concentrations of 10^{-4} and 10^{-5} M. IAA at concentration of 10^{-6} M has the same effects as TIBA at concentration of 10^{-3}M (Fig. 5.4B). Under warmer conditions, neither IAA nor TIBA could promote the stem elongation of the prairie plants (Fig. 5.4C, D). Application of these chemicals significantly inhibited stem elongation of the prairie ecotype. The effects of these chemicals can be seen as early as day eight of the growth period. Addition of IAA to TIBA treatments could rescued the stem elongation of both alpine and prairie ecotypes (data not shown).

Effects of exogenous plant growth regulators on the elongation of detached segments of alpine and prairie ecotypes of S. longipes

There were differences in stem segment elongation among stem segments placed under different temperature regimes and treated with different plant growth regulators (Fig. 5.5).

As for the alpine ecotype, there was no difference in stem elongation of segments placed under 10°/4°C and cultured in media supplemented with different concentrations of GA_3 and AMO 1618, indicating that segments grown under this condition were not sensitive to the application of either GA_3 or AMO 1618 (Fig. 5.5A). The effects of

exogenous plant growth regulators were more visible when segments were cultured under warmer temperature. Under 16°/10°C, there was no difference in segment elongations when segments were treated with AMO 1618 and GA_3. Similar effects were seen when segments were cultured under 22°/16°C. There was no difference in the segment elongation among treatments except when segments were cultured with 10^{-5}M GA_3. That concentration actually inhibited the elongation of segments of the alpine ecotype (Fig. 5.5A). AMO 1618 inhibited elongation of segments cultured under 28°/22°C, and adding GA_3 at concentration of 10^{-4}M to AMO 1618 actually could partially recover the inhibitory effects, implying that the inhibitory effect was due to the inhibition of GA_3. The application of GA_3 did not result in any difference in stem elongation of segments cultured under this condition.

Similar to the effects that we observed on the alpine ecotype, application of GA_3 and AMO 1618 had various effects on the elongation of the prairie segments (Fig. 5.5B). When segments were cultured under 10°/4°C, application of both AMO 1618 and GA_3 had no effects on the elongation. Neither application of GA_3 nor AMO 1618 promoted the elongation of the prairie ecotype segments grown under warmer conditions (Fig. 5.5B), indicating that GA_3 alone does not promote stem elongation of *S. longipes*. In fact, application of GA_3 and AMO 1618 in the same treatment actually inhibited elongation as seen in segments cultured under 28°/22°C (Fig. 5.5B).

Application of IAA and TIBA had a profound effect on elongation of segments of the alpine and prairie ecotypes (Fig 5.6). As for the alpine ecotype, under 10°/4°C, supplementation of IAA promoted stem elongation of segments. Application of TIBA did not have a profound effect. Similar effects were observed with segments cultured under

16°/10°C. There was no difference in IAA effects among treatments. Application of TIBA to segments cultured under this temperature regime resulted in neither inhibitory nor promoting effects on the elongation of these segments. Application of IAA to nutrient medium of segments cultured under warmer temperature regimes (22°/16°C and 28°/22°C) significantly promoted the elongation of the segments although there was no concentration as well as temperature effect. Application of TIBA to segments cultured under four different temperature regimes did not have a very strong inhibitory effect on the elongation of these segments (Fig. 5.6A).

IAA and TIBA had different effects on the prairie ecotype. Under 10°/4°C, application of IAA inhibited the elongation of segments and TIBA did not have an effect on the elongation of segments grown under this condition (Fig. 5.6B). The effects of these chemicals were more remarkable on plants grown under warmer temperature regimes. Under these temperature regimes, only IAA at concentration of 10^{-5} M could promote the segment elongation. Other IAA concentrations did not or had very little effect on the elongation of the prairie segments. TIBA, on the other hand, significantly inhibited the elongation of segments grown under these conditions (Fig. 5.6B).

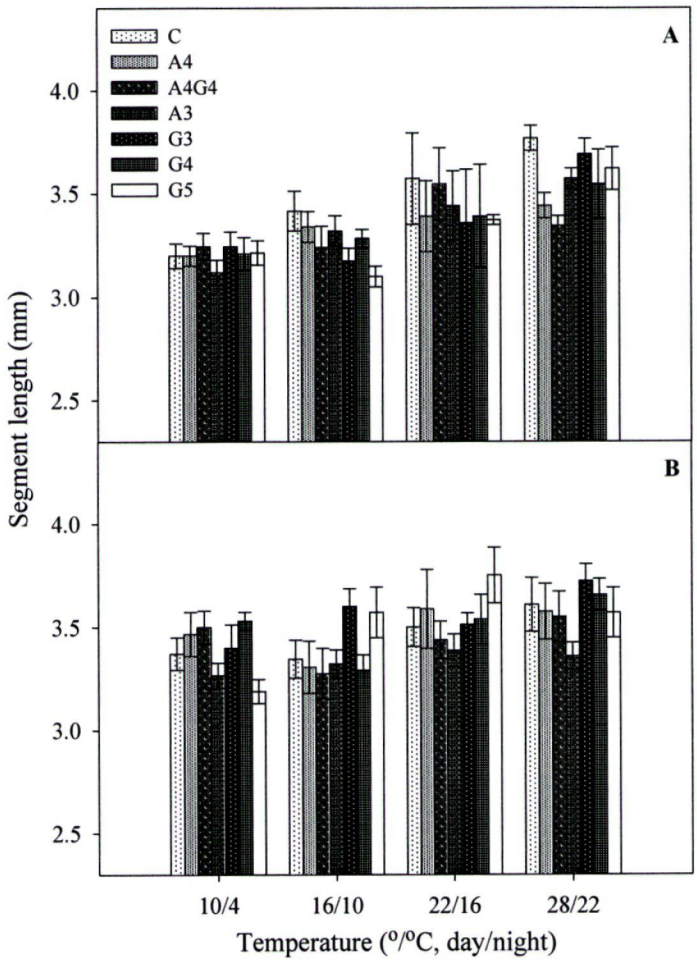

Figure 5.5. Segment elongation of alpine (A) and prairie (B) ecotypes of *Stellaria longipes* grown under four different temperature regimes and treated with different concentrations GA_3 and AMO 1618. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
G3:	GA_3 at concentration of 10^{-3}M
G4:	GA_3 at concentration of 10^{-4}M
G5:	GA_3 at concentration of 10^{-5}M
A3:	AMO 1618 at concentration of 10^{-3}M
A4:	AMO 1618 at concentration of 10^{-4}M
A4G4:	AMO 1618 at concentration of 10^{-4}M in combination with GA_3 at concentration of 10^{-4}M

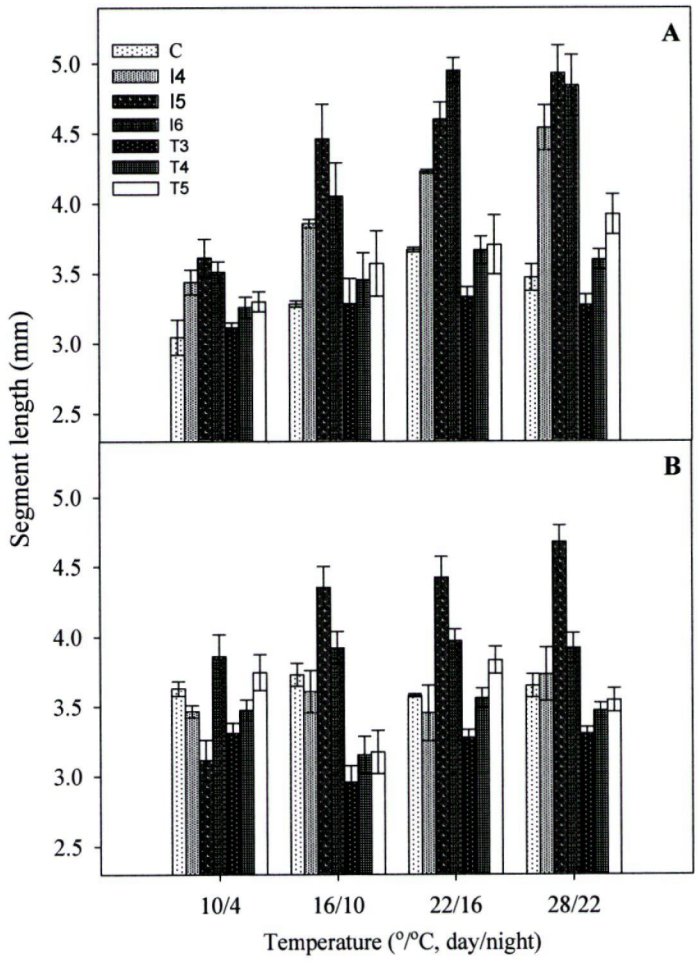

Figure 5.6. Segment elongation of alpine (A) and prairie (B) ecotypes of *Stellaria longipes* grown under four different temperature regimes and treated with different concentrations IAA and TIBA. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
I4	IAA at concentration of 10^{-4}M
I5	IAA at concentration of 10^{-5}M
I6	IAA at concentration of 10^{-6}M
T3	TIBA at concentration of 10^{-3}M
T4	TIBA at concentration of 10^{-4}M
T5	TIBA at concentration of 10^{-5}M

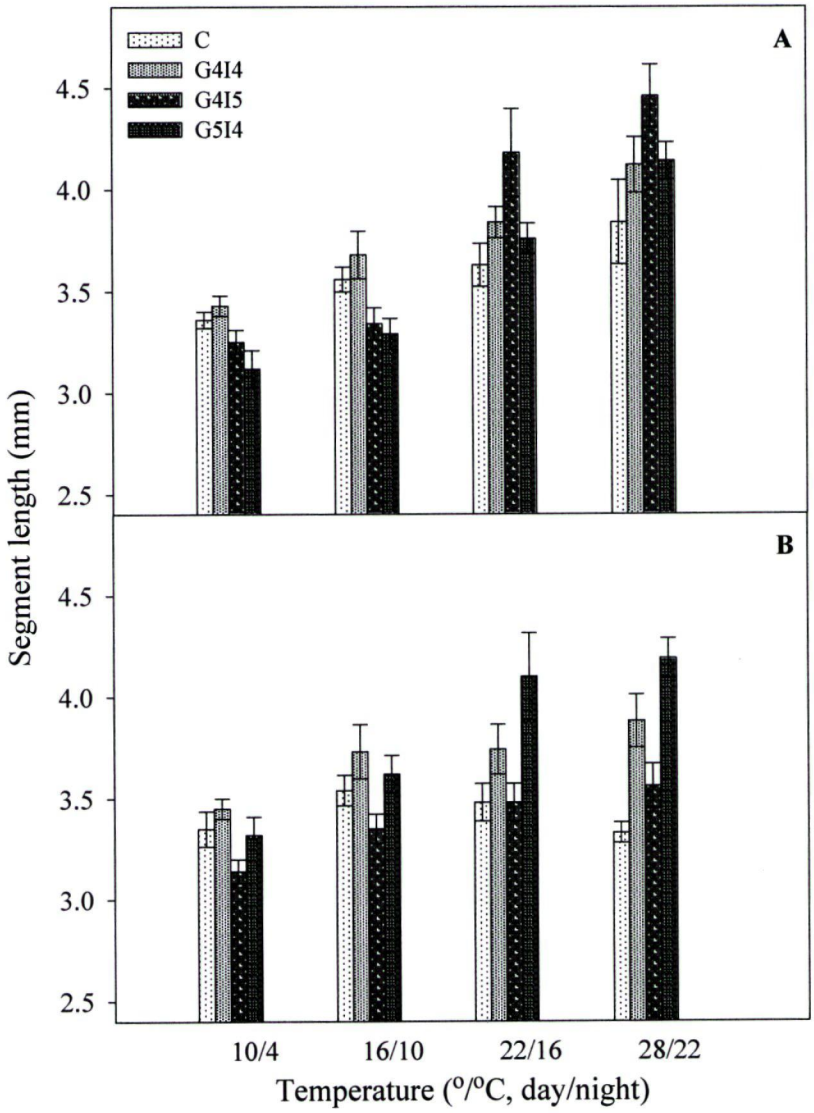

Figure 5.7. Segment elongation of alpine (A) and prairie (B) ecotypes of *Stellaria longipes* grown under four different temperature regimes and treated with different concentrations GA_3 and IAA. The error bars indicate standard error of the mean.

Abbreviations:

C:	Control treatment
G4I4	GA_3 at concentration of 10^{-4}M in combination with IAA at concentration of 10^{-4}M
G4I5	GA_3 at concentration of 10^{-4}M in combination with IAA at concentration of 10^{-5}M
G5I4	GA_3 at concentration of 10^{-5}M in combination with IAA at concentration of 10^{-4}M

Combination of different concentrations of GA_3 and IAA had different effects on the elongation of segments derived from alpine and prairie ecotypes (Fig. 5.7). As for the alpine ecotype, there was no difference in segment elongation when the same concentration of GA_3 and IAA were combined and added to segments cultured under 10°/4°C and 16°/10°C. Once the balance between these two hormones was broken due to changes in temperature regimes, the combination of these two hormones, one higher than the other, resulted in less elongation of segments grown under these conditions (Fig. 5.7A). When segments were cultured under warmer conditions, and when concentrations of GA_3 was equal to or higher than IAA, elongation of stem segments were significantly promoted. When IAA concentrations were higher, the elongation was inhibited as seen in segments grown under 10°/4°C, 16°/10°C and 22°/16°C (Fig. 5.7A).

These combinations of IAA and GA_3 did not result in the same effects on the prairie ecotype (Fig. 5.7B). Under 10°/4°C and 16°/10°C, there was no difference among control segments and segments cultured in media supplemented with GA4I4 (and G5I4. When there was less IAA in the medium (G4I5), the elongation of stem segments were significantly inhibited. Segments cultured under warmer temperature condition did not respond to the same way to applied GA_3 and IAA. There was no difference between applied hormone treatments although all of them promoted the segment elongation when segments were cultured under 22°/16°C. However, there was a difference in the response of segments cultured under 28°/22°C. Application of the same concentrations of IAA and GA_3 did not promote the segment elongation. However, the application of either lower or higher concentrations of IAA as compared to GA_3 had positive effects on the elongation

of segments derived from prairie ecotypes. There was no significant difference in elongation among segments grown under 22°/16°C and 28°/22°C (Fig. 5.7B).

5.4 Discussion

Two main points of interest arise from the this study: (i) the modifying action of PGRs on the effect of temperature, and (ii) the difference in the response of alpine and prairie ecotypes grown under the same conditions and treated with different plant growth regulators, and (iii) the possible interaction between GA_3 and IAA in modifying the effects of temperature on the stem elongation of alpine and prairie ecotypes.

Possible roles of exogenous plant growth regulators on stem elongation of the alpine and prairie ecotypes of S. longipes.

Some differences in sensitivity to GA_3 existed between ecotypes as indicated by response to applied GA_3. The alpine ecotype elongated in a similar magnitude as the prairie ecotype when they were grown under cold condition (10°/4°C). The alpine ecotype, however, appeared relatively insensitive to GA_3 even at very high concentrations when they were grown under warmer conditions, whereas, the prairie ecotype grown under the same condition responded very well. The difference in response of these two ecotypes to applied GA_3 changed followed temperature changes, suggesting that sensitivity of these ecotypes to GA_3 is strongly under temperature control. Therefore, the hypothesis that differences in sensitivity to applied GA_3 accounts for ecotypic differences in elongation of both ecotypes under different temperature regimes is supported. However, it should be noted that different environmental conditions for

growth may significantly affect the way in which *S. longipes* would respond to GA application (Macdonald et al., 1986).

Application of AMO 1618, GA biosynthesis inhibitor, resulted in a similar retardation of stem elongation in both ecotypes, whereas addition of GA_3 and AMO 1618 resulted in greater growth. The strong inhibitory effects of AMO 1618 and the slightly more than full recovery of plants when AMO 1618 was added in concert with GA_3 reflects the importance of GAs in stem elongation of the alpine and prairie ecotypes. Most of the evidence indicated that endogenous levels of GAs do not account for differences in stem elongation between these ecotypes grown under unstressed conditions. Levels of endogenous GAs along the growth curve were equal among ecotypes or higher in the shorter alpine ecotypes (Emery et al., 2001). Nevertheless, the general response of both ecotypes indicated that natural levels of GAs are an integral part of the stem elongation response to warm temperature and lengthening photoperiod.

Overall, the prairie and alpine ecotypes do have differential sensitivity of GAs. If prairie and alpine ecotypes had differential metabolism and production of GAs, it would be possible that GAs can be accountable for the relative dwarfness of the alpine ecotype compared to the rapid elongation of the prairie ecotype under warm temperature conditions. The results of this study should be considered preliminary with respect to the temperature effects and the stem elongation of *S. longipes*. The interaction between hormones and temperature stressed the importance of well-defined environmental conditions for studies of hormones' action (Heide, 1962).

Stem segments of both alpine and prairie ecotypes did not respond very well when cultured in medium supplemented with different concentrations of GA_3, suggesting

that GA_3 did not induce elongation in stem segments. When comparing the results of intact plants with those of detached segments, it can be concluded that GA_3 were not the only factor that regulate stem elongation of both alpine and prairie ecotypes *of S. longipes*. Galston and Kaur (1961) also noted the paradox that whereas GA treatment noticeably stimulates stem elongation in intact plants, it exerts little effect on the growth of the isolated segments. In many species, GA has been shown to play an essential role in the control of stem elongation in intact plants (Reid, 1987). Application of GA to plants was shown to neutralize the differences in stem elongation of plants grown under different temperature regimes (Tangeras, 1979; Moe, 1990; Grindal et al., 1998b). Grindal et al. (1998a) reported that altered stem elongation of pea in response to diurnal temperature alterations may be mediated by changes in the endogenous levels of GAs. Tonkinson et al. (1997) also reported that GA functions as a stimulus for continued cell extention by preventing cell maturation in the extension zone. As a consequence, higher GA levels in plants will increase the size of the extension zone and thus the ability of a plant to elongate under certain conditions. Stavang et al. (2005) suggested that although GA_1 levels do not change diurnally, sensitivity to Gas does and sensitivity to GA and thus stem elongation is dependent on both light and temperature. Temperature was shown to influence both the levels of bioactive GA levels and the ability of tissue to respond to GA (Pinthus et al., 1989). It was shown in my experiment that temperature influenced the ability of alpine and prairie ecotype tissue to response to GA, resulting in varied responses of these ecotypes to the same temperature and PGRs treatment.

There was a widely-held view that the external application of IAA has no appreciable effect on the elongation growth of intact plant stems (Trewavas, 1980). It was

suggested that IAA levels are not a limiting factor in the growth of intact plant stems and therefore do not function in the control of growth (Hanson and Trewavas, 1982). However, promotion of growth of stems of intact plants by the application of exogenous auxin was reported in several studies (Acton and Murray, 1974; Osborne, 1974). The promotion was rather slight with high concentrations of applied IAA. Application of IAA on intact stems of the alpine ecotype of *S. longipes* showed that IAA at low concentrations could promote stem elongation of the plants grown under 10°/4°C and 16°/10°C, suggesting that IAA has a significant role in the stem elongation of the alpine ecotype. In addition, application of IAA resulted in retardation of growth of the alpine ecotype grown under warmer conditions. It is possible that IAA had exceeded the optimal levels that plants need for induction of stem elongation. These high levels of IAA appeared to trigger feed back reactions that resulted in production of ethylene, which then inhibited plant growth. The results showed that low temperature might not be a favourable condition for the production of IAA, resulting in the short stems of alpine plants grown under this condition. Similar effects were observed in the prairie ecotype, suggesting that low temperature might inhibit the production of IAA, resulting in short stems. Application of TIBA inhibited growth, signifying that transportation of IAA strongly affected the stem elongation of both alpine and prairie ecotypes. Difference in the response of the alpine and prairie ecotypes suggested that they have different endogenous levels or sensitivity to IAA. In fact, it was reported that IAA levels increased two- to three-fold in shoots of the alpine genotype under low PAR (Kurepin et al., 2008). These two- to three-fold increases in IAA levels for the alpine ecotype under low PAR were also coincidently related with reduced ethylene production reported elsewhere

(Kurepin et al., 2006b), providing a partial explanation, at least, of the possible interaction between ethylene and IAA in controlling stem elongation of the alpine ecotype.

Application of IAA and TIBA to the detached segments of alpine ecotype showed that IAA promoted elongation of segments grown under all conditions. Application of IAA to segments grown under 16°/10°C could promote the elongation of segments to the extent of segments grown under warmer temperatures. This suggests that plants grown under this condition might not produce enough IAA that is necessary for stem elongation and that the temperature-induced elongation is related to auxin. This result was in accordance with the effects of applied IAA on intact plants. In addition, TIBA did not have significant effects on the detached segments although it inhibited the growth of intact plants, implying that inhibition of IAA transportation is very important in the response of both ecotypes to temperature. Law and Davies (1990) found that there was a close correlation between the endogenous levels of IAA and stem growth in a range of genetic lines of pea differing in height. Later, it was reported that exogenous auxin strongly promotes stem elongation, but that the promoting effect was short-lived (3-5h), and auxin subsequently became inhibitory (Yang et al., 1993). Data from my study support the results of Yang et al. (1993) who demonstrated that exogenous auxin strongly stimulates stem elongation in intact light-grown pea seedlings over a long term. Hall et al. (1985) also reported that IAA promotion of intact plant growth was similar to that of excised tissue segments even though the absolute amount of IAA absorbed by the tissues was appreciably higher in the later. Hall et al. (1985) proposed that stem growth is promoted by the application of IAA to intact plants, and that this occurs at a rate

comparable to that observed in excised stem segments. Firn (1982) also found that while most of auxin entered via the cut ends, it was the auxin entering radially via epidermis that caused the typical growth response. Hanson and Trewavas (1982) assumed that intact plants are not able to respond to exogenous IAA and proposed that growth in excised tissue segments largely represented an accelerated recovery from the injury caused by excision, which do not support my results. Both ecotypes responded well to application of IAA at both intact and detached levels. Since intact plants can respond to changes in IAA levels, changes in auxin concentration cannot be dismissed in discussion of growth regulation of these ecotypes. It is in accordance with Gray et al. (1998) who also showed that temperature-induced hypocotyl elongation (i) correlates with an increase in IAA levels, and (ii) depends on the auxin transport and response systems.

A recent report showed that exogenous auxin was capable of promoting mild increases in hypocotyl length in light-grown seedlings (Smallet et al., 1997). These increases were found to be caused by auxin mediated stimulation of ethylene biosynthesis and were not a direct effect of auxin treatment. In contrast to these findings, Gray et al. (1998) found that temperature-induced hypocotyls elongation is highly auxin-dependent and is unaffected by mutations in the ethylene response pathway, indicating that temperature-induced hypocotyl elongation involves a distinct regulatory pathway. Indeed, my results suggested that the increase in stem growth observed in plants grown under warm temperatures means temperature may directly regulate auxin levels to achieve this growth response in *S. longipes*. An alternate possibility is that temperature negatively regulates IAA catabolism. Although it is possible that the increase in IAA levels might be one of the effects of growth at high temperature rather than the actual mediator of the

response, evidence suggests that temperature-induced stem elongation is due to elevated endogenous auxin levels. The failure of exogenous auxin to promote the stem elongation of both ecotypes at low temperature may be that increased-auxin levels are necessary but not sufficient for increased cell elongation. Alternatively, the tissue specificity and timing of auxin synthesis and transport may be important factors for temperature induced stem elongation. The physical relevance of temperature-induced stem elongation is still unclear.

Possible interaction between IAA and GA_3 in controlling stem elongation of alpine and prairie ecotypes of S. longipes

Yang et al. (1996) provided strong evidence that IAA is a crucial regulator of stem elongation and that a substantial presence of IAA is necessary for moderate stem elongation to occur regardless of GA levels. Also, it was suggested that auxin cannot be solely responsible for controlling stem elongation. The combined effects of both IAA and GA are required, and a deficiency in either factor may lead to a dwarf genotype. My results showed that with the addition of exogenous IAA, applied GA manifested a specific growth response in elongating segments beyond the large response inducible by IAA alone (Brian and Hemming, 1958; Ockerse and Galston, 1967; Reid and Davies, 1992). It might be that IAA acted as the principal determinant of cell elongation, whereas GA contributes to stem growth primarily through a stimulation of cell division. Also, GAs and auxin cause different spatial distribution of elongation in internodes treated with GAs and auxin, providing further support for the differing roles played by GAs and auxin. It has been suggested that GA action must precede auxin action. However,

Kaufman et al. (1969) reported that IAA, at physiological concentrations, caused significant reduction in GA-promoted growth in excised oat stem sections. Kuraishi and Muir (1964) found that when GA was supplied in combination with IAA to dwarf pea, after apex excision, there was little or no effect of IAA on stem elongation.

Gibberellins and auxins are quite different growth promoting substances, and it has been proposed that the site of GA action is different from that of auxin action (Ockerse and Galston, 1967). The interaction of IAA and GA, as expressed through stem elongation, has been studied mainly in excised pea plants (Ockerse and Galston, 1967; Yang et al., 1996). In excised stem sections, a marked synergistic response has been observed between GA and IAA (Galston and Kaur, 1961; Okerse and Garlston, 1967). The relative lack of a response to GA of detached segments of both alpine and prairie ecotypes of *S. longipes* and the strong synergistic response induced by IAA and GA co-application imply that although GA cannot effectively induce growth in the absence of IAA, it enhances the elongation potential in the stem. Results of Yang et al. (1996) also indicated that GA-increased stem elongation potential may be additionally mediated by an enhancement of the auxin induction of cell elongation. Such a role of GA can be attributed to a spectrum of GA actions, including a facilitation of solute translocation to the elongation cells (Reid and Ross, 1993), an increase in the rate of wall relaxation and a reduction in the yield threshold (Cosgrove and Sovonick-Dunford, 1989; Behringer et al., 1990), and a decrease in wall stiffening by loosening the cross-linking between polymers (Potter and Fry, 1993).

Nevertheless, it should be noted that the absolute magnitude of the promotion of stem elongation was larger in the prairie ecotype than in the alpine. Stem growth of the

alpine ecotype could hardly be promoted by exogenous IAA as in the prairie. This indicated that stem elongation in intact plants of both ecotypes is not regulated solely by auxin. Auxin-induced growth in the intact stem appears to be essentially confined to an increase in cell elongation in the subtending internodes. Therefore, it is possible that stems of prairie ecotype that are endowed with a much higher number of cells showed a greater absolute elongation potential in the response to exogenous IAA than their dwarf counterparts.

In conclusion, it appears that stem growth in *S. longipes* is regulated at least by both auxin and GA. A synergistic effect between GA_3 and IAA in detached segments of both ecotypes indicated that the same mechanism of interaction may be present in an intact plant as in an excised stem. The mechanism of synergism has not been explained fully, but Hillman and Purves (1961) suggested that this might not be a direct influence of GA on IAA synthesis. Generally, it seems to be that GA leads to the production of an inhibitor which retards the auxin-destroying system and permites a sparing of IAA (Housley and Deverall, 1961). Growth might be dependent on an interaction between IAA and GA and that internal auxin is insufficient to permit maximum growth when GA_3 is applied. More work needs to be done to investigate the interaction between these two hormones in controlling stem elongation of *S. longipes.*

Chapter 6

General conclusion and perspectives

In the real world, plants have to respond to changes in the external environment, but they also have to maintain development when conditions fluctuate. Temperature is a good example of an environmental cue that provides both inductive and maintenance signals. Under certain circumstances developmental pathways and the resulting physiological responses are manipulated by the thermal stimulus. The present project explored some cellular and physiological aspects of stem elongation of the alpine and prairie ecotypes of *S. longipes* grown under different temperature regimes. Some conclusions can be drawn from this work:

- Temperature exerted different effects on the growth of alpine and prairie ecotypes. Variation in the elongation of both ecotypes under the same conditions is most likely due to differences in their ability to respond to specific temperature regimes. Such differences could be due to the inheritance of regulatory genes acquired by these plants to enhance their survival or adaptation in different habitats. The profound difference in response of both ecotypes to different temperature regimes makes these ecotypes a good model system to explore the key morphological differences that underlie the response.
- Stem elongation of *S. longipes* is a complicated process that involves cell division and cell elongation. Both cortical and epidermal cells regulated the elongation response and the epidermal cells played a more significant role. In addition, correlation between the thickening of epidermal cell walls and inhibition of stem elongation suggested that epidermal cells play an important role in controlling the

rate and timing of elongation. Overall, difference in the timing of cell wall thickening, deposition of phenolic compounds and cross-linking of cell wall material and especially the dynamic of cortical MTs might caused differences in the elongation, division and stretching ability of the cells, resulting in the difference in the stem elongation of alpine and prairie ecotypes of *S. longipes* grown under contrasting temperature regimes.

- Significant differences in the levels of ethylene produced by the alpine and prairie ecotypes grown under different temperature conditions. Low temperature regimes inhibited growth in the prairie plants through elevated ethylene levels. High temperature regimes retarded growth in the alpine plants due to high stress-induced ethylene formation. Such different ethylene productions can account for the difference in stem elongation of two ecotypes, which responded differently to the same temperature regimes. Much of our knowledge of ethylene synthesis and response comes from crop species grown under different temperature or light conditions. This finding adds up to those previously reported on effects of wind stress, light quality and quantity on stem elongation of a wild species, *S. longies*.
- It also appears that stem growth in *S. longipes* is regulated at least by both IAA and GA_3. A synergistic effect between GA_3 and IAA in detached segments of both ecotypes indicated that the same mechanism of interaction may be present in an intact plant as in excised stem. The mechanism of synergism has not been explained fully, but Hillman and Purves (1961) suggested that this might not be a direct influence of GA_3 on IAA synthesis. The most satisfactory explanation seems to be that GA_3 leads to the production of an inhibitor which retards the

auxin-destroying system and permits a sparing of IAA (Housley and Deverall, 1961). Growth might be dependent on an interaction between IAA and GA and that native auxin is insufficient to permit maximum growth when GA_3 is applied. Results showing the possible interaction between GA_3 and IAA provide a good bridge for the examination of a possible interaction between hormones in controlling stem elongation plasticity,particularly interesting in this wild species.

Temperature influences the rate of photosynthesis or production of high energy compounds. Many factors, including light, temperature, carbon dioxide, etc. influence the rates of plant growth. As a result, temperature should not be considered in isolation and therefore any statement about an optimal temperature for growth of a species cannot be made. It is possible that temperature-induced stem elongation of alpine and prairie ecotypes may be a mechanism by which plants can direct elongation growth during different periods of a year to maximize stem elongation at critical times to compete effectively in a plant community. The response of stem and cell elongation to temperature is an observation, which leads to questions concerning the ecological or evolutionary basis for temperature response. The results of the present study demonstrated that along with light, temperature plays a significant role in maintaining and amplifying the stem elongation response of alpine and prairie ecotypes of *S. longipes*. Differences in the magnitude of stem elongation, cellular structures, ethylene levels, sensitivity to applied plant growth hormones were probably due to their original contrasting habitats. Although the results did not precisely show what particular hormones and the molecular mechanisms responsible for the variation in stem elongation within the alpine and prairie ecotypes under varied temperature regimes, the results did provide some insight to the

cellular structure and possible roles of microtubules as well as plant growth regulators on the stem elongation. The data also showed some interesting directions that may provide a basis for the future researches, which include:

- Studying the temperature effects on stem elongation on plants grown under different light conditions, such as different PAR conditions or different Red/Far Red ratio to determine the possible interaction between temperature and light in regulating the stem elongation of alpine and prairie ecotypes of *S. longipes.*
- Studying the endogenous levels of major plant growth regulators, especially auxin and gibberellins, to complete the physiological data of temperature-induced stem elongation response.
- Studying the regulatory effects or synergistic roles of plant growth regulators on plants at cellular levels to see how these substances regulate the stem elongation of plants grown under different temperature regimes structurally.

Rapid progress towards revealing the complex regulatory mechanisms that control plastic responses of alpine and prairie ecotypes of *S. longipes* will only be achieved if there is better collaboration between further physiological, developmental and molecular approaches.

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