

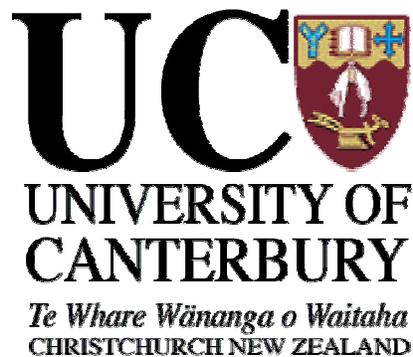
**An examination of the relationship between  
NO, ABA and auxin  
in lateral root initiation and root elongation in tomato**

**A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Plant Biotechnology**

**By**

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**at the**



**2006**

This research work is dedicated to my beloved parents.

## ABSTRACT

The length of the primary root and the density of lateral roots determine the architecture of the root. In this thesis the effect of NAA, ABA and the NO donor SNP alone as well as the combination of ABA or NAA with SNP on lateral root development was investigated. The interaction between CPTIO, a NO scavenger, and NAA or SNP is also reported.

Following preliminary experiments in which it was observed that the aerial part of the seedling influenced LR growth and that there was a possible inhibitory effect of light on cultured root tips, experiments were conducted with excised roots tips in the dark. NAA was shown to have the potential to initiate LRs across a wide concentration gradient with the total number of LRs and initiated lateral root primordia (LRP) remaining constant across the range of concentrations tested. Over the last decade, nitric oxide (NO), a bioactive molecule, has been reported to be involved in the regulation of many biological pathways. The presence of NO in the system provided via sodium nitroprusside (SNP), promoted LRP initiation based on the NAA concentration gradient; but without changing the total LR initiation, that is LRs plus primordia density remained constant along the concentration gradient of NAA. The absence of LR and LRP in the treatments of CPTIO (a NO scavenger) with SNP or NAA suggests that NO regulates LRP initiation triggered by NAA, which is in agreement with the recent paper published after the commencement of this study (Correa-Aragunde et al., 2006).

In agreement with previous studies, ABA inhibited lateral root development by reducing LR density and the number of LRs. The experiments with fluridone, an ABA biosynthesis inhibitor, may indicate that endogenous ABA was at sufficient concentrations in the excised root tips to inhibit primordia initiation. In this study, evidence is presented for the first time to show that SNP can relieve the inhibitory effect of ABA on LR density and number of LRs suggesting the NO, released from SNP, acts downstream of ABA.

Overall these data confirm a critical role for NO in LR initiation.

# CONTENTS

<b>LIST OF FIGURES</b>	i
<b>LIST OF TABLES</b>	ii
<b>LIST OF PLATES</b>	iii
<b>ABBREVIATIONS</b>	iv
<b>CHAPTER 1.0</b>	
<b>INTRODUCTION</b>	1
<b>1.1 Overview</b>	1
<b>1.2 Structure and formation of a lateral roots (LRs)</b>	2
<b>1.3 Factors affecting lateral root initiation and elongation</b>	6
1.3.1 Environmental factors	6
1.3.2 Hormones	8
1.3.2.1 Auxin and LR initiation/elongation	8
1.3.2.2 Abscisic acid (ABA) and LR initiation/elongation	10
1.3.2.3 Cytokinin and LR initiation/elongationGibberellins	11
1.3.2.4 Gibberellins and root initiation/elongation	12
1.3.2.5 Ethylene and root initiation/elongation	12
<b>1.4 Nitric Oxide (NO)</b>	13
1.4.1 NO in animal kingdom	13
1.4.2 Involvement of NO in plants	14
<b>1.5 Measurements and analysis of results</b>	16
1.5.1 Measurements from the experiments	16
1.5.2 Density of LRs	17
<b>1.6 Aims and objectives</b>	17

## CHAPTER 2.0

<b>MATERIALS AND METHODS</b>	19
<b>2.1 Materials</b>	19
2.1.1 The plant material	19
2.1.2 Sources of plant growth regulators and other chemicals	19
2.1.3 Media	20
<b>2.2 Methods</b>	20
2.2.1 Preparation of basal medium	20
2.2.2 Sterilization	20
2.2.3 Seed germination	21
2.2.4 The effect of ABA on seedling roots and excised root tips	21
2.2.5 The effect of SNP on seedling roots and excised root tips	24
2.2.5.1 The effect of SNP on seedling roots in a growth room with 14 h of light and 10 h of darkness	24
2.2.5.2 The effect of SNP on excised root tips in complete darkness	24
2.2.5 Interaction between ABA and SNP on excised root tips of tomato	24
2.2.7 The effect of NAA on excised root tips of tomato	25
2.2.8 The effect of fluridone on excised root tips of tomato	26
2.2.9 Interaction between NAA and SNP on excised root tips of tomato	27
2.2.10 The effect of CPTIO on NAA and SNP on excised root tips of tomato	28
2.2.11 Image and data analysis	29
2.2.11.1 Image scanning	29
2.2.11.2 Statistical analysis	29

## CHAPTER 3.0

<b>RESULTS</b>	30
<b>3.1 Morphology of seedlings and an excised root tip grown in White's medium</b>	30
<b>3.2 Development of lateral root primordia</b>	31
<b>3.3 The effect of ABA on seedling roots and excised root tips</b>	32
<b>3.4 The effect of SNP on seedling roots and excised root tips</b>	36
3.4.1 The effect of SNP on seedling roots in a growth room with 14h of light and 10h of darkness	36
3.4.2 The effect of SNP on excised roots in a growth room with 14h of light and 10h of darkness	41
3.4.3 The effect of SNP on excised root tips in complete darkness	42
<b>3.5 Interaction between ABA and SNP on excised root tips of tomato</b>	47
<b>3.6 The effect of NAA on excised root tips of tomato</b>	54
<b>3.7 The effect of fluridone on excised root tips of tomato</b>	59
<b>3.8 Interaction between NAA and SNP on excised root tips of tomato</b>	65
<b>3.9 The effect of CPTIO on SNP and NAA on excised root tips of tomato</b>	78

## CHAPTER 4.0

<b>DISCUSSION</b>	88
<b>4.1 Preliminary experimental studies</b>	88
4.1.1 Seedling roots versus excised root tips	88
4.1.2 The effect of initial length of the root segments	89
4.1.3 The effect of light on LR elongation	90
<b>4.2 Confirmation of published data</b>	90
4.2.1 ABA inhibits LR emergence	91
4.2.2 SNP promotes LR density	92
4.2.3 NAA promotes LR initiation and elongation	92
<b>4.3 Interaction between NAA and NO</b>	93

<b>4.4</b>	<b>NO operates downstream of ABA</b>	94
<b>4.5</b>	<b>The effect of CPTIO on SNP and NAA</b>	95
<b>4.6</b>	<b>Conclusions</b>	97
<b>4.7</b>	<b>Directions for future study</b>	98
<b>CHAPTER 5.0</b>		
	<b>REFERENCES</b>	100
<b>CHAPTER 6.0</b>		
	<b>APPENDICES</b>	109
	<b>Appendix 1 – Preparation of White’s medium</b>	109
	<b>Appendix 2 – Preparing the ABA solution</b>	111
	<b>Appendix 3 – Preparing the SNP solution</b>	112
	<b>ACKNOWLEDGEMENTS</b>	113

## LIST OF FIGURES

<b>Figure 1.1</b>	Diagrammatic illustration of LRP formation in <i>Arabidopsis</i>	5
<b>Figure 2.1</b>	Typical diagram to illustrate the measurements of different parameters on a seedling and an excised root tip	23
<b>Figure 3.1</b>	The effects of ABA on 4-day-old, 2 cm long seedling roots and 4-day-old, 2 cm long root tips of tomato, cultured at $25\pm 1^{\circ}\text{C}$ in complete darkness.	33
<b>Figure 3.2</b>	The effect of SNP on 3-day-old, 1 cm long tomato seedling roots in a growth room with a daily photoperiod of 14 h of light and 10 h of darkness.	37
<b>Figure 3.3</b>	The effects of SNP on 4-day-old, 2 cm long excised root tips of tomato cultured in complete darkness.	43
<b>Figure 3.4</b>	Interaction between ABA and SNP on LR density.	49
<b>Figure 3.5</b>	Interaction between ABA and SNP on the number of LRs.	50
<b>Figure 3.6</b>	Interaction between ABA and SNP on the PR length.	51
<b>Figure 3.7</b>	Interaction between ABA and SNP on the length of the longest LR.	52
<b>Figure 3.8</b>	Interaction between ABA and SNP on apical distance.	53
<b>Figure 3.9</b>	The effect of NAA on 4-day-old, 2 cm long excised root tips of tomato.	55
<b>Figure 3.10</b>	The effect of fluridone on 4-day-old, 2 cm long tomato excised root tips.	60
<b>Figure 3.11 (i)</b>	Interaction between NAA and SNP on LR density.	67
<b>Figure 3.11 (ii)</b>	Interaction between NAA and SNP on primordia density.	68
<b>Figure 3.11 (iii)</b>	Interaction between NAA and SNP on LR plus primordia density.	69
<b>Figure 3.12 (i)</b>	Interaction between NAA and SNP on the number of LRs.	70
<b>Figure 3.12 (ii)</b>	Interaction between NAA and SNP on the number of primordia.	71
<b>Figure 3.12 (iii)</b>	Interaction between NAA and SNP on the number of LR plus primordia.	72

<b>Figure 3.13</b>	Interaction between NAA and SNP on the PR length.	73
<b>Figure 3.14</b>	Interaction between NAA and SNP on the length of the longest LR.	74
<b>Figure 3.15 (i)</b>	Interaction between NAA and SNP on LR apical distance.	75
<b>Figure 3.15 (ii)</b>	Interaction between NAA and SNP on primordia apical distance.	76
<b>Figure 3.15 (iii)</b>	Interaction between NAA and SNP on LR plus primordia apical distance.	77
<b>Figure 3.16</b>	The effect of CPTIO on SNP and NAA on the LR, primordia and LR plus primordia density.	80
<b>Figure 3.17</b>	The effect of CPTIO on SNP and NAA on the number of LRs, primordia and LR plus primordia	82
<b>Figure 3.18</b>	The effect of CPTIO on SNP and NAA on the PR length.	84
<b>Figure 3.19</b>	The effect of CPTIO on SNP and NAA on the length of the longest LR	85
<b>Figure 3.20</b>	The effect of CPTIO on SNP and NAA on the LR, primordia and LR or primordia apical distance	86

## LIST OF TABLES

<b>Table 3.1</b>	The effects of SNP on 3-day-old, 1 cm long excised root tips of tomato incubated in a growth room with 14 h of light and 10 h of darkness. The values are means $\pm$ SE from 3 independent experiments (n=16).	41
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## LIST OF PLATES

<b>Plate 2.1:</b>	Tomato seedlings germinated for four days in darkness at $25\pm 1^{\circ}\text{C}$	21
<b>Plate 3.1:</b>	Morphology of seedlings and an excised root tip grown in White's medium	30
<b>Plate 3.2:</b>	Lateral root primordia	31
<b>Plate 3.3</b>	Photographs of 2 cm long excised tomato root tips cultured for 7 days in complete darkness at $25\pm 1^{\circ}\text{C}$ .	42
<b>Plate 3.4</b>	Photographs of 2 cm long tomato root tips incubated for 7 days in different concentrations of NAA in darkness at $25\pm 1^{\circ}\text{C}$ .	54
<b>Plate 3.5</b>	Photographs of 2 cm long excised root tips of tomato treated with NAA or SNP and together with CPTIO	78

## ABBREVIATIONS

ABA	-	Abscisic acid
C	-	Celsius
CPTIO	-	2-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF-2 DA	-	4,5-diaminofluorescein diacetate
dH <sub>2</sub> O	-	Distilled water
g	-	gram
H	-	Hour
L	-	Litre
LR	-	Lateral root
LRP	-	Lateral root primordia
M	-	Molar
mg	-	milligram
Min	-	Minutes
ml	-	millilitre
mm	-	millimeter
M.W	-	Molecular weight
NAA	-	1-naphthylacetic acid
NO	-	Nitric oxide
NR	-	Nitrate reductase
NOS	-	Nitric oxide synthase
PR	-	Primary root
SNP	-	Sodium nitroprusside
Soln	-	Solution
μM	-	Micro molar

# CHAPTER 1

## INTRODUCTION

### 1.1 Overview

Roots are of equal importance to shoots as the life support system of plants. In addition to providing anchorage, roots help to absorb water, facilitate the extraction of micro- and macro-nutrients and transport nutrients to the aboveground parts. Also roots are one of the sites that synthesise many metabolites that are essential for a plant (Dubrovsky et al., 2006). The length of the primary root (PR) and the density of lateral roots (LRs) determine the architecture of the root system (Malamy and Benfey, 1997). Three major processes that affect the overall architecture of the root system are: (1) cell division at the primary root meristem that enables continuous growth by adding new cells to the root, (2) LR formation, which increases the exploratory capacity of the root system, and (3) root hair formation, which increases the total surface of the PR and LRs (Lopez-Bucio et al., 2003). The anatomy of the root is very consistent throughout the higher plants, but the number, placement and the direction of growth of each root in the system are highly variable, even among genetically identical plants (Malamy, 2005). The focus in this thesis is on lateral roots.

Lateral root initiation is influenced by complex interactions among different hormonal and environmental factors. The number and location of LRs are not predetermined: each plant integrates information from its own environment and this influences root initiation and elongation (Malamy and Ryan, 2001). For example, availability of nutrients plays an important role in both the number and the placement of LRs (Leyser and Fitter, 1998). In soil or a medium with patchy nutrient distribution, LRs preferentially proliferate in the nutrient-rich zone (Drew et al., 1973; Drew and Saker, 1975; Robinson, 1994).

Lateral root initiation and elongation is also under hormonal control. Genetic and physiological evidence suggests that auxin plays an important role at several specific developmental stages in LR formation (Casimiro et al., 2003). Overproduction of auxin or application of exogenous auxin leads to increased numbers of LRs (Blakely et al., 1988; Celenza et al., 1995; Boerjan et al., 1995; Himanen et al., 2004). Further studies showed that endogenous nitric oxide (NO) is also involved in LR initiation, suggesting a possible interaction of NO with auxin (Correa-Aragunde et al., 2004; Correa-Aragunde et al., 2006).

Another plant hormone, abscisic acid (ABA), has been shown to affect LR development by inhibiting LR initiation (De Smet et al., 2003). The latest studies show that the inhibitory effect of ABA is likely to be mediated by an auxin-independent pathway (De Smet et al., 2003).

Root growth is an important component of plant growth but has received little attention from plant breeders because of difficulties associated with observing roots *in situ*. Understanding the factors affecting LR formation and their interactions is crucial for modulating the architecture of the root system. The ability to modulate root architecture helps to overcome the inability of plants to move towards water or nutrients and thus maximize crop production. However, the mechanisms by which plants incorporate these factors into LR initiation and elongation are poorly understood. In this thesis the focus is on the interaction between auxin, NO and ABA.

## 1.2 Structure and formation of lateral roots (LRs)

Most of the higher plants have three types of roots. They are the main root (tap root), lateral root and adventitious root (Fitter, 1991). The first root to emerge from a germinated seed is the radicle which, in most dicotyledons, enlarges to form a prominent [main root](#). But in monocots, the radicle is short-lived and is replaced by a mass of adventitious roots (<http://www.ffp.csiro.au>).

Dicotyledonous plants produce one or more orders of lateral root branches. The main root produces the first order laterals and these produce the second order laterals and so on. The different orders of roots vary in many ways, for example, in their thickness, branching patterns, growth rates, capacity for secondary growth, life spans and structural features. These variations will influence their capacity to obtain water and nutrients, support mycorrhizal associations and survive adverse conditions. Higher order lateral LRs are generally thinner, shorter and do not live as long as those of lower orders (<http://www.ffp.csiro.au>). The meristem of the main root is formed during embryogenesis, whereas in many plants, the meristems of laterals and adventitious roots are formed post-embryogenically (Malamy and Benfey, 1997). However, in some groups such as the Cucurbitaceae, lateral root primordia (LRP) are initiated during embryogenesis (Dubrovsky, 1986).

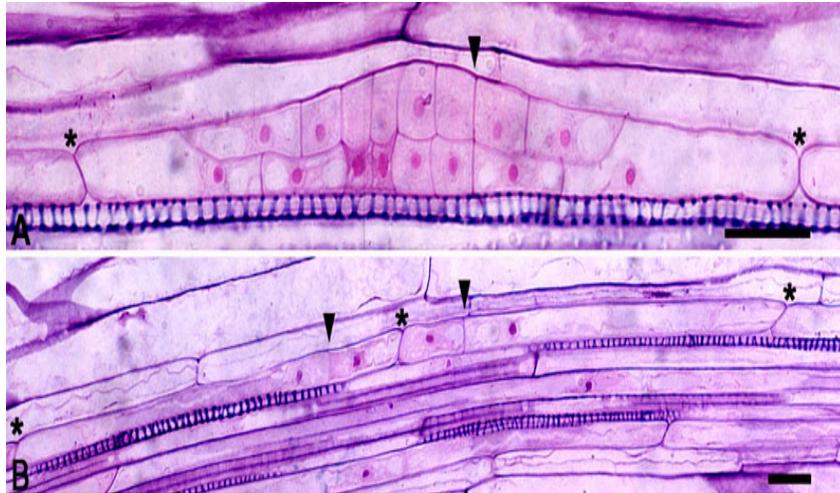
The process of LR development takes place in three steps. These steps are initiation, organisation and emergence. In this thesis, measurements of elongation incorporated emergence has been evolved. Many studies have been done on LR development, but the basic mechanisms that control LR initiation are poorly understood (Dubrovsky et al., 2006). The exact mechanism varies between each plant species, but the basic features are common to all species. In most plants LRs are initiated from a single layered pericycle. Generally the pericycle is the exterior cell layer of the provascular cylinder which is surrounded by the ground meristem (cortex + endodermis) and epidermis, but in cucumber, the pericycle becomes multilayered (Dubrovsky, 1986). In some Pteridophytes, a second tissue, the endodermis participates in lateral root primordial

(LRP) formation (Lin and Raghavan, 1991). Rarely, the cortex can also participate in LRP formation. In *Arabidopsis thaliana*, which has a simple root anatomy composed of single layers of epidermal, cortical and endodermal cells surrounding the vascular tissues, LRP are derived from the pericycle. LR initiation in tomato is similar to that in *Arabidopsis* (Laskowski et al., 1995).

Lateral root primordia can be initiated in two different ways. In *Arabidopsis* and some other angiosperms, a “longitudinal bicellular” type of LRP initiation was first discovered. In this type, two adjoining cells along a cell file opposite to the xylem poles become founder cells. Another type of initiation, the “longitudinal unicellular” type, occurs when only one cell along the file becomes a founder cell and participates in primordium formation (Dubrovsky et al., 2001). However, longitudinal unicellular type of LRP initiation is rarely found (Blakely et al., 1982). Pericycle founder cells are described as “cells that acquire a developmental fate different from that of their mother and, as a consequence, play a principal role during the first stages of LR initiation” (Dubrovsky et al., 2000).

Initially, the two pericycle founder cells within the same cell file undergo almost simultaneous polarized asymmetric transverse divisions and create two short cells flanked by two longer cells in *Arabidopsis* (Laskowski et al., 1995). These cells undergo symmetrical and asymmetrical division and create a group of short cells that are similar in length as described in Figure 1.1 b (Casimiro et al., 2001; Dubrovsky et al., 2001). A similar series of mitotic division also occurs in both pericycle cell files followed by radial expansion (Casimiro et al., 2003). The central short daughter cells divide several times periclinally in the meristematic zone and form a dome-shaped LRP. These cells rapidly elongate and differentiate towards the base within the parent root, giving rise to an organised primordium (Malamy and Benfey, 1997; Casimiro et al., 2003). This LRP forms a functional meristem and elongates, causing the LR to emerge through the epidermis. LR emergence appears to be due to expansion of existing cells rather than cell division (Malamy and Benfey, 1997). Organisation into a LRP requires sufficient cell

divisions so that there are at least three pericycle founder cell layers (Dubrovsky et al., 2001).



**Figure 1.1** Diagrammatic illustration of LRP formation in *Arabidopsis*. Two types of lateral root primordium initiation in *Arabidopsis thaliana*. **A**, unicellular longitudinal; LRP cells are enclosed in a cell wall of the founder cell 6-8 mm from the root tip. **B**, Longitudinal bicellular. Earliest stage of LRP initiation on a histological section of the root portion 2-4 mm from the root tip. Note, the first asymmetrical division in two pericycle cells leading to LRP formation. Anticlinal cell walls of the founder cells are marked with asterisks. Location of the cell wall resulting from the first division of founder cells is marked by arrowhead. Black bar represent 20  $\mu\text{m}$ . (Dubrovsky et al., 2001, reproduced with permission of Springer-Verlag, cited in <http://3e.plantphys.net/article.php?ch=e&id=275>.)

As described above, in most plants LRs are initiated at the protoxylem poles. In tomato, primordia initiated either opposite to the protoxylem pole or slightly to one or other side of it, which gives a diarch pattern (Barlow and Adam, 1987). But the position of LRs along the main root is poorly understood. Since the spacing between the LRs is regular, it has been suggested that there must be a positional mechanism that causes regular spacing

(Charlton, 1991). Further study has shown that the presence of LRP guarantees the initiation of the other primordium in a different pole (Michael et al., 2006).

Generally, LRs are initiated acropetally along the root, where the primordia are initiated distal relative to the already initiated primordia. However, earlier stages of LRP initiation can be found basipetally in the roots, where the primordia are initiated proximal relative to the already initiated primordia (Dubrovsky et al., 2000). Primordia are initiated at a certain distance behind the apex and this distance remains almost constant as the PR elongates. Therefore primordium initiation advances acropetally in tomato (Barlow and Adam, 1987).

### **1.3 Factors affecting lateral root initiation and elongation**

There are two different mechanisms or pathways that determine the architecture of the root system: an “intrinsic pathway” and a “response pathway”. The former is essential for organogenesis and growth that determines the characteristic architecture of the plant. The latter determines how plants respond to external signals and modulates the intrinsic pathway (Malamy, 2005). The intrinsic determinants determine LR initiation, the developmental pattern of the primordium, LR formation and growth. However, the morphology of the root system is also dramatically influenced by environmental signals (Forde and Lorenzo, 2001; Lopez-Bucio et al., 2003).

#### **1.3.1 Environmental factors**

Environmental factors such as nutrients, water, light, temperature, soil texture, pH and gravitropic signals have significant influence on root architecture (Dubrovsky and Rost, 2003). Soil nutrients are critical elements for plant growth and productivity. The ability of a plant to respond appropriately to nutrient availability is essential for it to adapt to the environment (Lopez-Bucio et al., 2003). Nutrients such as nitrate, phosphate, sulphate and iron act as signals and activate molecular mechanisms that modify cell division and cell differentiation processes within the roots which, eventually, change the root

architecture. Nutrients such as nitrate and phosphate have an important role in LR elongation. Zhang and Forde (1998) have shown that an increasing nitrate concentration reduces PR elongation but does not cause any change in the LR density because the number of LRs are also reduced. The  $\text{NO}_3^-$  signal appears to have two distinct effects. Exposure of *Arabidopsis* roots to a high concentration of nitrate (10 mM) retarded the elongation of LRs. However, when a split root system was used and when the plant was grown on a low nitrate concentration (10  $\mu\text{M}$ ), but with a part of the root system exposed to a high nitrate concentration (10 mM), LR elongation was promoted only on the low  $\text{NO}_3^-$  side. This suggests that the inhibitory effect is a response to a nitrate sufficiency (Zhang and Forde, 1998). Studies on *Arabidopsis* have shown that high concentrations of phosphate (P) increase PR length and no change in the number of LRs results in a reduction in LR density (Linkohr et al., 2002). A moderate concentration of P (100  $\mu\text{M}$ ) results in more LR growth than PR growth. Low sulphate concentrations increased the number of LRs (Kutz et al., 2002).

The response of root architecture to nutrients can be modified by plant growth regulators, suggesting that the nutritional control of root elongation may be mediated by hormones (Lopez-Bucio et al., 2003). The gravity vector appears to be an important factor in controlling the position of the PR and LRs. The initial growth of LRs is nearly horizontal in orientation. But when the LRs elongate, they change to a near-vertical position (Mullen and Hangarter, 2003). This change in orientation coincides with the increase in growth rate (Mullen et al., 2005).

Light is an important factor which controls growth and differentiation of plant cell, tissue and organ cultures. Many studies show that light controls *in vitro* root formation. Light stimulates root formation in rhizome fragments of *Helianthus tuberosus* (Gautheret, 1969). Light has different effects on root initiation and root elongation (Seko and Nishimura, 1996). For instance, light stimulates root initiation and inhibits root elongation in wheat (Vinterhalter et al., 1990). However, transfer of plants from darkness to light induces elongation after a short lag period. The reverse transfer from light to darkness

efficiently arrests root elongation but promotes the appearance of LR<sub>s</sub> (Vinterhalter et al., 1990).

### **1.3.2 Hormones**

LR development is also under hormonal control. Since most hormones exist at some basal level in plants under a growth situation, hormonal effects on root system architecture can be considered to be intrinsic. However, hormones are also important components of response pathways, since their levels can be adjusted in response to environmental signals. Malamy (2005) suggests that “overlapping hormone signalling pathways provide the complexity that is constantly integrating information from many sources into a decision to initiate a LR”. There are many different hormones involved in LR initiation and elongation.

#### **1.3.2.1. Auxin and LR initiation/elongation**

Among the plant hormones, auxin is the only substance that is actively distributed throughout the entire plant. Auxin is biosynthesised typically in young, proliferating parts of the shoot and is more abundant in shoots than in roots (Ljung et al., 2001; Ljung et al., 2005). It is transported basipetally down the plant, that is, basipetally down the shoot and acropetally to the root tip (Bhalerao et al., 2002). Accumulated auxin in the root tip is transported basipetally towards the elongation zone of the root (Rashotte et al., 2000; Casimiro et al., 2001; Ottenschlager et al., 2003). Indole-3-acetic acid (IAA), phenyl acetic acid (PAA), a chlorinated form of IAA (4-Cl-IAA) and indole-3-butyric acid (IBA) are some of the naturally occurring compounds that show auxin-like effects (Ludwig-Muller and Cohen, 2002).

Auxin plays an important role in LR initiation and elongation. Synthetic auxin such as 1-naphthylacetic acid (NAA) and 2,4-D-related compounds have been used commercially for many years. In several experiments on different plants, a similar auxin effect on LR

elongation was found. Increasing the auxin level resulted in a significant increase in the number of LRs (Blakely et al., 1998). A recent study showed that exogenous auxin increased the probability of initiating LRP within the same vascular pole from two to six times (Michael et al., 2006). Internal auxin overproduction by transgenic plants also resulted in an increased number of LRs (Boerjan et al., 1995; Laskowski et al., 1995). Casimiro et al. (2001) confirmed the effect of auxin on LRP initiation by growing *Arabidopsis* seedlings with an auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA), which reduced the number of primordia initiated by inhibiting the auxin transportation from shoot to root tip (Casimiro et al., 2001). In addition, LR initiation was decreased in an auxin-insensitive mutant, *aux 1* (Casimiro et al., 2003).

Auxin regulates all three stages of LR development. In the presence of exogenous auxin, at any developmental stage, a LRP can develop into a LR. However, in the absence of exogenous auxin, only LRP that have reached the stage of having at least 3-5 cell layers can develop into LRs. There was no development observed in the LRP which had fewer than three cell layers (Laskowski et al., 1995). These observations suggest that the development of LRP from 3-5 cell layers is auxin-dependent and beyond this stage, they are either auxin-independent or they produce the required amount of auxin themselves. Experiments with an aberrant lateral root (*alf3-1*) mutant show the importance of auxin for LR initiation beyond the 3-5 cell layer stage (Celenza et al., 1995).

Elongation of the first LR coincides with the emergence of the first true leaves. Experiments with *Arabidopsis* seedlings showed that young seedlings required shoot-derived auxin to develop LRs. As the root system develops, mature seedlings are less dependent on leaf-derived auxin and start to produce their own auxin (Bhalerao et al., 2002). This was further supported by a recent study, of both the roots of seedlings and excised roots of *Arabidopsis*, showing that auxin synthesis rates and levels in excised roots are very much less than those of seedlings. In addition, it strongly suggests that developing LRs can supply auxin at later developmental stages (Ljung et al., 2005).

The removal of the apical segment of the main root prior to the formation of the first true leaves had little effect on LR initiation but inhibited LR emergence, suggesting that auxin also regulates LR emergence (Bhalerao et al., 2002).

Auxin interacts with other hormones and nutrients in most kinds of developmental processes. Reduction of LR initiation with a high C:N ratio was correlated with the accumulation of auxin in the hypocotyls (Malamy and Ryan, 2001). However, the addition of exogenous auxin overcame this nutritional effect, suggesting that nutritional cues were not blocking the ability of the root to respond to auxin (Malamy and Ryan, 2001). Therefore, it is possible that the reduction of LR initiation could be due to a blockage in the movement of auxin from the shoot to root.

Studies in the past mostly concentrated on the effect of auxin in its regulation of root initiation and elongation. However, there is little information about the influence of auxin on the other aspects of root architecture, such as the length of LRs and apical distance.

### **1.3.2.2 Abscisic acid (ABA) and LR initiation/elongation**

ABA regulates normal root initiation and elongation. A study showed that endogenous ABA may inhibit LR initiation but it is possible that ABA affects different processes of root development differently, via differing sensitivities to ABA (Hooker and Thorpe, 1998). An exogenous supply of ABA inhibits LR initiation by blocking the activation of LR meristems at the 3-5 cell layer of LRP development. However, this inhibition is reversible in the absence of ABA (De Smet et al., 2003). Exogenous application of auxin failed to rescue this ABA-induced arrest, suggesting that the inhibitory effect of ABA is mediated by an auxin-independent pathway (De Smet et al, 2003). A recent study on legumes has shown that ABA exerts an opposite effect on LR elongation in legumes. It reduced LR density by reducing the number of LRs elongated in non-legumes but promoted LR density by increasing the number of LRs elongated in legumes regardless of whether they were nodulating or non-nodulating legumes (Liang and Harris. 2005).

Hooker and Thorpe, (1998) found that the application of fluridone, an ABA biosynthesis inhibitor, promoted both the number of emerged LR and LRP in the presence of ABA on tomato excised root tips. Furthermore, they showed that ABA is involved in the regulation of apical dominance in roots.

Considerable evidence suggests that ABA interacts with other signalling pathways. It interacts with ethylene to control shoot and root growth under water stress (Sharp and LeNoble, 2002). ABA interacts with NO and ethylene in guard cells (Neill et al., 2002; Garcia-Mata and Lamattina, 2002; Desikan et al., 2004; Tanaka et al., 2005). Recently it has been shown that ABA interacts with auxin signalling pathways in roots (Rock and Sun, 2005). In addition, ABA is required to mediate the regulatory effects of nitrate on root branching. The inhibitory effect of high  $\text{NO}_3^-$  was reduced in ABA-insensitive mutants, suggesting that the inhibitory effect of  $\text{NO}_3^-$  may operate through an ABA signal transduction pathway in mediating the nitrate effect on LR elongation (Signora et al., 2001). However, the effect of ABA on LR initiation and elongation is poorly understood. The interaction of ABA with NO in guard cells suggests that there is a possibility that internal NO could regulate the effect of ABA on LR elongation.

### **1.3.2.3 Cytokinin and LR initiation/elongation**

Cytokinins are synthesised both in shoot and root, but mainly in the root. It is transported to shoots via the xylem (Aloni et al., 2005), although it could also be transported from shoots to roots via the phloem (Gessler et al., 2004). Reduction in the cytokinin level in early LRP development suggests a potential role for cytokinin in LR initiation (Lohar et al., 2004). Many researches have found that, unlike auxin, cytokinin inhibits LR initiation (Hinchee and Rost, 1986). Further study with cytokinin deficient transgenic *Arabidopsis*, which over expressed cytokinin oxidase/dehydrogenase (*AtCKX*) gene and increased cytokinin breakdown confirmed the opposite function in the regulation of shoot and root elongation, that is, reduced shoot apical meristems and leaf primordia growth and increased root initiation (Werner et al., 2003). A recent study on rice by Debi et al. (2005)

shows that the effect of cytokinin varies depending on developmental stages of LR formation. This effect is restricted to initiation of LRP. They found that no inhibitory effect was evident on LR elongation from the primordia that had already formed. Galis et al. (2005) further supported the inhibitory effect of cytokinin in LR initiation in transgenic tobacco plant. Activity of *AtCKX* promoter was observed in the region where primordia developing and this is supporting the previous finding that cytokinin exerts an inhibitory role in the LR initiation (Galis et al., (2005). Cytokinin not only inhibits LR initiation but also promotes LR elongation by increasing cell length (Debi et al., 2005).

Cytokinin, together with auxin, plays an essential role in the formation of roots, shoots and their relative growth (Skoog and Miller, 1957 in Werner et al., 2001). The inhibitory effect of cytokinin on LR initiation and promotion in elongation can be overcome by the addition of exogenous auxin, suggesting that cytokinin acts on an auxin-dependent pathway (Debi et al., 2005). This finding agrees with the previous studies that the auxin:cytokinin ratio plays an important role in LR elongation (Hinchee and Rost, 1986)

#### **1.3.2.4 Gibberellins and root initiation/elongation**

Gibberellins (GA) are important regulatory factors in the control of shoot and root growth (Richards et al., 2001). GA is biosynthesised mainly in hypocotyls and root tips. The requirement of GA for normal root growth was revealed by the use of chemical inhibitors and mutants of GA biosynthesis. Experiments with mutations such as *d5* in maize and *gib-1* in tomatoes showed that root elongation is slower in these mutants than in the wild type (Baluska et al., 1993). However, GA is ineffective on its own, although it regulates ethylene-mediated growth responses in shoots and roots (Steffens et al., 2006). GA, together with ethylene, promotes the number of roots and the growth rate of emerged roots.

### **1.3.2.5 Ethylene and root initiation/elongation**

Ethylene is also involved in the growth and differentiation of shoots and roots. During flooding, more ethylene is produced and it promotes LR formation (Yamamoto et al., 1995). A decrease in the ethylene level with the application of an ethylene biosynthesis inhibitor reduced the number of adventitious roots produced by *Rumex palustris* affected by flood (Visser et al., 1996). Aloni et al. (2006) have found that ethylene also mediates cluster root formation under iron deficiency. Ethylene interacts with auxin signalling pathways. Moreover, elevated ethylene concentration might interrupt polar auxin transport (Visser et al., 1996).

Ethylene is also involved in the effect of ABA on root growth. The inhibitory effect of ABA requires a functional ethylene-signalling cascade (De Smet et al., 2003). However, ethylene production is not the purpose of ABA action; rather, ABA inhibits ethylene synthesis in order to maintain root growth at a low water potential (Sharp, 2002).

## **1.4 Nitric Oxide (NO)**

NO is a gaseous free radical which serves as a signal in plants and animals. The biological significance of NO was recognised by *Science* in 1992 which named NO the “Molecule of the year” (Koshland and Koshland, 1992). Initially it was thought to be a toxic by-product of oxidative metabolism. Later it was revealed that it regulates many cellular functions. NO has been more comprehensively studied in animals than in plants, even though it was first discovered in a plant (Klepper, 1979).

### **1.4.1 NO in animal kingdom**

In mammals, NO is an intercellular and intracellular signalling molecule with a broad spectrum of regulatory functions in the central nervous system, in platelet inhibition, in programmed cell death, in host response to infection and in cardiovascular and immune

systems (Moncada et al., 1991; Lloyd-Jones and Bloch, 1996). It is also involved in oxidative stress in ageing and age-related diseases (Balaban et al., 2005). Nitric oxide synthase (NOS) is responsible for the primary source of NO, which has three different isomers: eNOS (endothelial NOS), nNOS (neuronal NOS) and iNOS (inducible NOS) (Alderton et al., 2001). NOS oxidizes L-arginine to NO and citrulline.

#### **1.4.2 Involvement of NO in plants**

Although NO research in plants is not as advanced as in animals, in the last decade NO has been shown to participate in many physiological processes and it has become an increasingly popular target of investigation in plants. NO synthesis in plants appears more complex than in mammals. It is synthesised via both nitrite- and arginine- dependent mechanisms. Initially high NO emission was correlated with high nitrite levels and nitrate reductase (NR) activation (Rockel et al., 2002). However, more recently several sources that contribute to NO-mediated responses have been suggested (Corpas et al, 2004). Nitrite-mediated sources are NR, mitochondria, the apoplast, nitrate-NO reductase and class-2 haemoglobin. The arginine-mediated source is NOS (AtNOS1), which is different from known animal NOS protein (Guo, 2003). In addition, NO is also released from soils and the amount varies depending on temperature, oxygen availability, pH and N-fertilisation rates (Stohr and Stremlau, 2006).

The NO donor, sodium nitroprusside (SNP) was shown to induce LR formation in maize (Gouvea et al., 1997), tomato seedlings (Correa-Aragunde et al., 2004) and adventitious root formation in cucumber plants (Pagnussat et al., 2002). The effect was dose-dependent and the optimum concentration of SNP varied depending on plant species and the experimental conditions. For example, the maximum biological response was at 10  $\mu$ M SNP for adventitious root formation in cucumber hypocotyls and 200  $\mu$ M SNP for LR formation in tomato seedlings.

A study published after this project commenced showed that NO modulates the expression of cell cycle regulatory genes in tomato pericycle cells resulting in induced LR initiation (Correa-Aragunde et al, 2006). A further study with 2-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) demonstrated that NO was required for LR initiation, but not for LR emergence (Correa-Aragunde et al., 2006).

Gouvea et al. (1997) reported that NO had no effects on IAA-induced cell expansion, but, as mentioned above NO interacts with IAA in adventitious root elongation in cucumber plants (Pagnussat et al., 2002). Furthermore, a transient increase in NO concentration occurred during adventitious root initiation following treatment with IAA, suggesting a role for NO in auxin-signalling pathways. Anatomical studies examined the formation of adventitious root primordia in the IAA or SNP treatments, whereas none were found in the control (Pagnussat et al., 2004). The results of recent experiments carried out by adding CPTIO on different days to 0.1  $\mu\text{M}$  NAA-treated tomato seedlings suggest that NO was required for cell cycle progression and establishment of LRP in the pericycle but not for the elongation and emergence of LRs (Correa-Arangundae et al., 2006). However, this study did not consider both LRP and LRs together in relation to the effect of NAA or SNP. In addition, a similar effect on primordia development was reported in either 0.1  $\mu\text{M}$  NAA or 200  $\mu\text{M}$  SNP. It would be interesting to find the relationship between SNP and different concentrations of NAA on both LR initiation and elongation.

Different mechanisms have been suggested for the involvement in stomatal closure via ion channels and chemical messengers (Schroeder et al., 2001). More recently, it has been suggested that NO accumulation in guard cells was necessary for the ABA-induced stomatal closure, placing a new component in the ABA signalling transduction pathway. This was further confirmed by the external application of ABA to guard cells, which led to increased accumulation of NO. Treatments of guard cells with a NO scavenger, CPTIO, inhibited stomatal closure whereas treatment with a NO donor, SNP, increased stomatal closure (Garcia-Mata and Lamattina, 2002; Neill et al., 2002). The interaction of ABA with NO in guard cells suggests that there is a possibility that internal NO could regulate the effect of ABA on LR elongation.

NO can be monitored by various techniques such as electron paramagnetic resonance and NO electrodes (Crawford and Guo, 2005). NO-sensitive fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA) allows the detection of the presence of NO in both animals and plant cells (Kojima et al., 1998; Foissner et al., 2000). DAF-2 DA is hydrolysed in living cells by cytosolic esterases to release 4,5-diaminofluorescein (DAF-2) which reacts with NO to produce the fluorescent triazole derivative triazofluorescein (DAF-2T), giving rise to increased green fluorescence. Further confirmation can be done with 4-aminofluoresceindiacetate (4-AF DA) which lacks one of the amino groups that constitutes the NO specific domain of the DAF-2 DA molecule. Cessation of increased levels of green fluorescence with 4-AF DA confirms that the green fluorescence corresponds to an accumulation of endogenous NO, and not to unspecific reaction of the probe (Garcia-Mata and Lamattina, 2002). Although the advantages of this technique are obvious, the variations of DAF-2T and DAF-2 are hard to control in living objects (Stohr and Stremlau, 2006). They are highly dependent on pH, the availability of oxygen, the presence of antioxidative substance and of  $\text{Ca}^{2+}$  (Zhang et al., 2002). However, such mechanisms were beyond this project.

Several lines of experiments will be needed to obtain a better understanding of NO interaction with the phytohormones. The identification of genes that are responsible for the production of NO could lead to the discovery of new signalling functions for many metabolic enzymes. In this thesis, a source of NO (SNP) as well as a limited supply of a NO scavenger (CPTIO) was used.

## **1.5 Measurements and analysis of results**

### **1.5.1 Measurements from the experiments**

In recent decades, either data obtained from various experiments on seedlings or excised roots have been presented in different ways. Some researchers considered only the percentage of seedlings or excised roots with LRs as a function of different concentrations of hormones (Creus et al., 2005). Some researchers considered the number

of LRs, which were greater than 0.5 mm while others considered LRs greater than 1 mm (Hooker and Thorpe, 1998). Some other experiments considered both the number of primordia and the number of LRs in order to find the effect of hormones on LR initiation and elongation. Most of the experiments considered the PR length but only a few articles considered the length of the longest LR.

### **1.5.2 Density of LRs**

LR density is customarily calculated as a ratio of the number of LRs to the PR length. Most studies in the past did not include LRP in their analysis, where LRP have the potential to develop as a LR (Lopez-Bucio et al., 2003). This method of estimation is applicable only when in all ecotypes and at all ages, the ratio between the root portion covered by LRs and the PR length remains constant. This happens vary rarely and therefore, it is necessary to evaluate the LR density in terms of the portion of the PR where LRs are present (Dubrovsky et al., 2006).

### **1.6 Aim and objectives**

NO appears to be involved in most aspects of plant growth, development and in response to both biotic and abiotic environmental system. In this research the aim was to examine whether NO, ABA and NAA interact to control LR initiation and elongation. In the present study, several objectives were pursued using seedling roots and excised root tips of tomato. They were:

1. To determine if tomato seedling roots or excised root tips were a suitable model for studying LR initiation and elongation by incubating seedlings and root tips in White's media (control treatment).

2. To determine if ABA inhibits LR initiation and/or elongation by monitoring the response to an ABA biosynthesis inhibitor
3. To determine if NO operates downstream of NAA in LR initiation and elongation by supplying a source of NO, SNP.
4. To determine if NO operates down stream of ABA in LR elongation by supplying a source of NO, SNP.
5. Confirm that NO is a key component of the signal transduction of NAA and ABA by incubating root tips in media containing the NO scavenger, CPTIO.

### **Hypothesis**

NO is a critical downstream component of the signal transduction pathway leading to LR initiation and the effects of NAA and ABA are mediated by NO.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials

##### 2.1.1 The plant material

A 25 g packet of untreated tomato seeds (*Lycopersicon esculentum* Mill. cv. Money maker) were purchased from Eco Seeds, Christchurch, New Zealand and stored at 4°C. Some experiments (2.2.6, 2.2.8, 2.2.9 and 2.2.10) were carried out with seeds purchased from Asian Seeds, Taradale, Napier, New Zealand.

##### 2.1.2 Sources of plant growth regulators and other chemicals

Plant growth regulators and chemicals used in this work and their sources are listed below.

<b>Plant growth regulators</b>	<b>Source</b>
(±) cis, trans- abscisic acid (99%) (ABA)	Sigma <sup>®</sup> Tissue culture reagent, St.Louis, USA
1-Naphthylacetic acid (98.5%) (NAA)	BDH Chemicals Ltd, Poole England.
<b>chemicals</b>	
Fluridone	Duchefa Biochemie, Netherlands
Sodium nitroprusside (99.0%) (SNP)	BDH Chemicals Ltd, Poole England.
Chromium trioxide	BDH Chemicals Ltd, Poole England.
CPTIO	Sigma <sup>®</sup> , St.Louis, USA

### **2.1.3 Media**

White's modified medium supplemented with White's organics (Butcher and Street, 1964) was used for all experiments.

## **2.2 Methods**

### **2.2.1 Preparation of basal medium**

Stock solutions of major salts, White's organic supplement and iron solutions were made and stored at 4°C (Appendix 1). The required volumes of the stock solutions were mixed and sucrose (8 g/l) was added to the medium. Then the pH was adjusted to 5.7±0.1.

Required volume of media for each treatment was dispensed into each Schott bottle (500 ml) and sterilised.

### **2.2.2 Sterilisation**

Media used for plant tissue culture work were sterilised by wet autoclaving, which is wet heat sterilisation of 20 min at 121°C and 120 kPa. Petri dishes, filter paper and pipette tips were sterilised by dry autoclaving where dry air is used for 20 min at 121°C and 120 kPa. Solutions such as NAA and ABA were filter-sterilised using an ultra-filtration unit Millex<sup>®</sup> GP, Millipore Corporation (22 µm) under aseptic conditions. SNP, CPTIO and fluridone were also filter-sterilised. Scalpels and forceps were sterilised in 100% ethanol and left to dry before and after transferring root tips to each of eight Petri dishes. Tomato seeds were surface sterilised in a laminar flow work station for 10 min with 5% (v/v) household bleach containing 4.8% (w/v) sodium hypochlorite as an active agent. Then they were thoroughly rinsed three times with sterile distilled water.

### 2.2.3 Seed germination

Surface sterilised seeds were transferred to sterile Petri dishes (30 seeds per dish) containing one layer of filter paper soaked with 8 ml of sterile distilled water (Plate 2.1). After sowing, seeds were incubated at  $25\pm 1^\circ\text{C}$  in the dark.



**Plate 2.1:** Tomato seedlings germinated for four days in darkness at  $25\pm 1^\circ\text{C}$

### 2.2.4 The effect of ABA on seedling roots and excised root tips

Fresh ABA stock solution (0.264 M) was prepared by dissolving 13.21 mg of ABA with several drops of 1 N NaOH, added to  $\text{dH}_2\text{O}$ , and then the pH was adjusted to  $5.7\pm 0.1$  before it was made up to 50 ml with  $\text{dH}_2\text{O}$ . The required amount of the ABA stock solution to be transferred to each bottle of medium was calculated. See Appendix 2 for the calculations. In a laminar flow cabinet under aseptic conditions, ABA stock solution was filter-sterilised before it was added to each bottle of the medium. For control, no ABA solution was added. Each bottle of autoclaved basal medium was dispensed

(approximately 25 ml each) into 16 sterile Petri dishes. ABA concentrations tested in this experiment were 0.1 and 1  $\mu\text{M}$ .

Apical root segments each of two cm long were excised from 4-day-old tomato seedlings and transferred to media in eight Petri dishes (90 mm diameter). Two root tips were placed in each dish. Sixteen replicates per treatment were prepared. Similarly, 2 cm long seedlings were chosen and transferred to media in eight Petri dishes. Transferring of apical segments or seedlings was carried out randomly. All the Petri dishes were wrapped along the edge using polyethylene film. Both excised root tips and seedlings were incubated for seven days in darkness at  $25\pm 1^\circ\text{C}$ .

After seven days, the number of LRs which emerged ( $>1$  mm long) from each root tip was counted and all the Petri dishes were scanned for further analysis. The PR length, the apical distance (the length from the root tip to the most apical lateral root) and the length of the longest LR were measured as described in Figure 2.1 a and b, using Image Pro. LR density (the number of lateral roots per cm of the portion of primary root axis where LRs are present) was calculated as follows:

$$LR \text{ density} = \frac{\text{No of LRs} \times 10}{(\text{Length of PR} - \text{LR apical distance})}$$

This experiment was repeated at least three times and the mean value was taken as the final result. Data were analysed as described in Section 2.2.11.2.



## **2.2.5 The effect of SNP on seedling roots and excised root tips**

### **2.2.5.1 The effect of SNP on seedling roots in a growth room with 14 h of light and 10 h of darkness**

The concentrations of 50, 100 and 300  $\mu\text{M}$  SNP and control were chosen to perform this experiment. Three-day-old seedlings were used in this experiment. Stock solution (59.58 M) was prepared by dissolving 0.5958 g of SNP in 10 ml  $\text{dH}_2\text{O}$ . The required amount of this solution to be transferred to each bottle of autoclaved basal medium was calculated. See the Appendix 3 for the calculations. The rest of the experiment was carried out exactly the same as described in Section 2.2.4 but 1 cm long apical segments and 1 cm long seedling roots were used. All the Petri dishes were kept in a growth room under 14 h of light:10 h of dark photoperiod at  $25\pm 1^\circ\text{C}$ . Light period ( $60 \mu\text{mol}/\text{m}^2/\text{s}$ ) was given by white fluorescent tubes (Philips, the Netherlands). After five days of treatment, measurements were taken as described in Section 2.2.4.

### **2.2.5.2 The effect of SNP on excised root tips in complete darkness**

A range of concentrations of SNP (50, 100, 300 and 500  $\mu\text{M}$ ) was chosen in addition to the control (no SNP). The experiment was carried out as described in Section 2.2.4, but with excised root tips only. After seven days, measurements were taken as described in Section 2.2.4.

## **2.2.6 Interaction between ABA and SNP on excised root tips of tomato**

The following grid was designed to test the effect of combinations of different ABA and SNP concentrations. ABA and SNP stock solutions were prepared and filter-sterilised under aseptic conditions. Eight bottles of media (containing 200 ml each) were prepared and autoclaved. The required amount of a stock solution to be transferred to each bottle

was calculated and transferred under aseptic conditions. Each bottle of media was dispensed into eight sterile Petri dishes. Apical root segments (each 2 cm long) were excised from 4-day-old seedlings and transferred to Petri dishes. All the Petri dishes were incubated at  $25\pm 1^\circ\text{C}$  in complete darkness. After seven days, measurements were taken as described in Section 2.2.4.

	<b>ABA</b> $\longrightarrow$	<b>0</b>	<b>1 <math>\mu\text{M}</math></b>
<b>SNP</b> $\downarrow$	<b>0</b>	1	2
<b>500 <math>\mu\text{M}</math></b>		3	4
<b>1000 <math>\mu\text{M}</math></b>		5	6
<b>2000 <math>\mu\text{M}</math></b>		7	8

### 2.2.7 The effect of NAA on excised root tips of tomato

This experiment was carried out with 50, 100, 500 nM NAA and a control (without NAA). NAA stock solution (0.186 M) was prepared by dissolving 18.6 mg of NAA with several drops of 1 N NaOH and the pH was adjusted to  $5.7\pm 0.1$ . This solution was made up to 100 ml with dH<sub>2</sub>O. The required amount of this solution to be transferred to each bottle of autoclaved medium was calculated. The rest of the experiment was carried out exactly the same as described in Section 2.2.5.2. After seven days of treatment, measurements were taken as described in Section 2.2.4.

### 2.2.8 The effect of fluridone on excised root tips of tomato

Fluridone stock solution (6 M) was prepared by dissolving 9 mg in 1.5 ml of 100% ethanol. Therefore sterilisation was not necessary for this solution. Two different controls in addition to 1 and 0.1  $\mu$ M fluridone were prepared for this experiment. Neither fluridone nor ethanol was added to one of the controls. The equal volume of 100% ethanol as fluridone stock solution was added to another control in order to check whether the ethanol used to dissolve fluridone might have any influence on the result. The experiment was carried out as described in Section 2.2.5.2. After seven days, measurements were taken as described in Section 2.2.4.

After the root measurements were completed the roots were soaked in 5% (W/V) chromium trioxide for 5 min and the number of lateral root primordia (LRP) was counted using a light microscope. Emerged LRs that were less than 1 mm long were also counted as primordia. Primordia apical distance (the length from the root tip to the most apical primordium) was measured as described in Figure 2.1 (b). LR plus primordia apical distance was also measured (the length from the root tip to the most apical LR or primordium).

Primordia density and lateral plus primordia density was calculated as described in Hooker and Thorpe (1998) and the formulae used are shown below:

$$\text{Primordia density} = \frac{(\text{Number of primordia}) \times 10}{(\text{Length of primary axis} - \text{Primordia apical distance})}$$

$$\text{Lateral plus primordia density} = \frac{(\text{Number of laterals} + \text{Number of primordia}) \times 10}{(\text{Length of primary axis} - \text{The shortest length of either apical distance or primordia apical distance})}$$

LR density was calculated as described in Section 2.2.4.

### 2.2.9 Interaction between NAA and SNP on excised root tips of tomato

The following grid was designed to test the effect of the combinations of different concentrations of NAA and SNP:

	SNP →	0	500 μM
NAA ↓	0	1	2
	50 nM	3	4
	100 nM	5	6
	500 nM	7	8
	1000 nM	9	10

SNP and NAA stock solutions were prepared (Sections 2.2.5.1 and 2.2.7) and filter-sterilised under aseptic conditions. Ten bottles of basal media (containing 200 ml each) were prepared and autoclaved. The required amount of a stock solution to be transferred to each bottle was calculated and transferred under aseptic conditions. The medium in each bottle was dispensed into eight sterile Petri dishes. Apical segments (each 2 cm long) were excised from 4-day-old seedlings and transferred to Petri dishes. All the Petri dishes were incubated at  $25\pm 1^{\circ}\text{C}$  in complete darkness. After seven days, measurements were taken as described in Section 2.2.4. The number of LRP was counted as described in Section 2.2.5.

#### **2.2.10 The effect of CPTIO on NAA and SNP on excised root tips of tomato**

The following experiment was designed in order to test the effect of CPTIO on SNP and NAA. The treatments of SNP (500  $\mu\text{M}$ ), NAA (50 nM), CPTIO (1 mM) and control, in addition to the mixture SNP with CPTIO and NAA with CPTIO were carried out in this experiment. SNP stock solution was prepared by dissolving 0.05959 g of SNP in 10 ml  $\text{dH}_2\text{O}$  and NAA stock solution was prepared by dissolving 15.52 mg in 2 l  $\text{dH}_2\text{O}$ . CPTIO stock solution was prepared using 26.2 mg in 10 ml  $\text{dH}_2\text{O}$ . All the stock solutions were filter-sterilised under aseptic conditions. Six bottles of basal media (containing 25 ml each) were prepared and autoclaved. The required amount of a stock solution to be transferred to each bottle was calculated and transferred under aseptic conditions. The medium in each bottle was dispensed into five glass vials (5 ml each). Apical segments (each 2 cm long) were excised from 4-day-old seedlings and transferred to glass vials (each with a total capacity of 25 ml). One root tip was transferred to each glass vial and five replicates were prepared for each treatment. All the glass vials were incubated at  $25\pm 1^{\circ}\text{C}$  in complete darkness. After seven days, measurements were taken as described in section 2.2.4. Then LRP was counted as described in section 2.2.8.

## **2.2.11 Image and Data analysis**

### **2.2.11.1 Image Scanning**

All samples were scanned using ScanMakerX12 USL (MICROTEK, China) and ScanWizard 5.85 (Microtek International, Inc) as the software.

### **2.2.11.2 Statistical analysis**

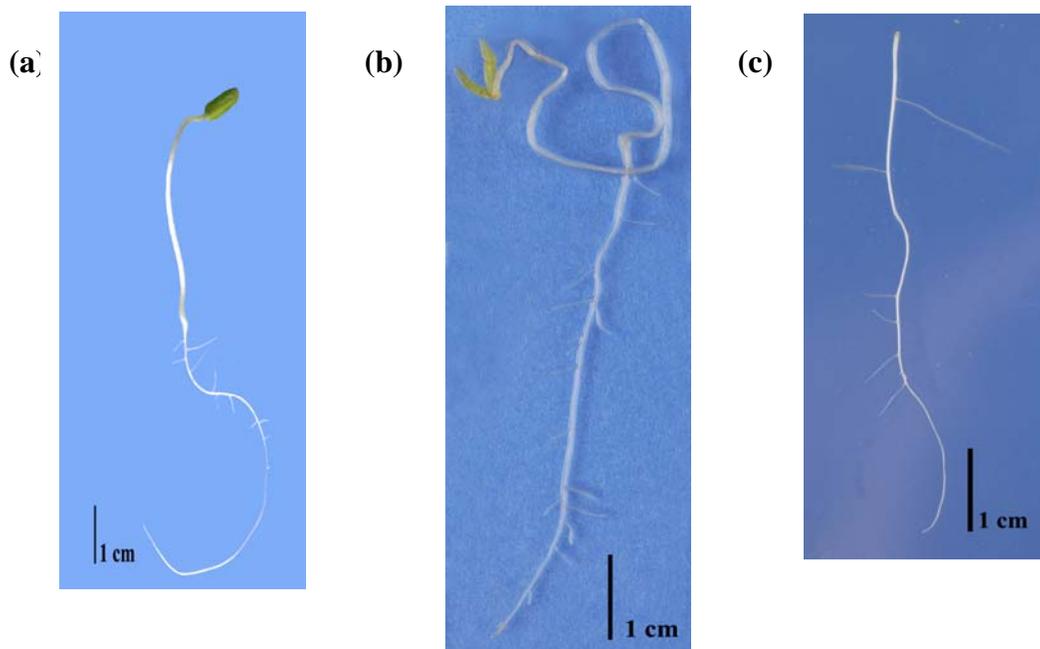
Data were analysed according to standard error of the mean, where data were grouped from replicate experiments. One-way ANOVA was performed to test whether the treatment groups differed significantly. Tukey's least significant difference test was conducted using Statistix 8 (Microsoft Corporation, Seattle, USA) to perform multiple comparisons for determining which mean scores were significantly different.

## CHAPTER 3

### RESULTS

#### 3.1 Morphology of seedlings and an excised root tip grown in White's medium

Photographs of seedlings and an excised root tip grown in culture media (control) with 14 h of light and 10 h of darkness (Plate 3.1 a) or complete darkness (Plates 3.1 b and c) are shown below. Seedlings exposed to light developed a thick rigid stem, roots and green leaves, whereas seedlings cultured in complete darkness were etiolated and had soft stem and roots.

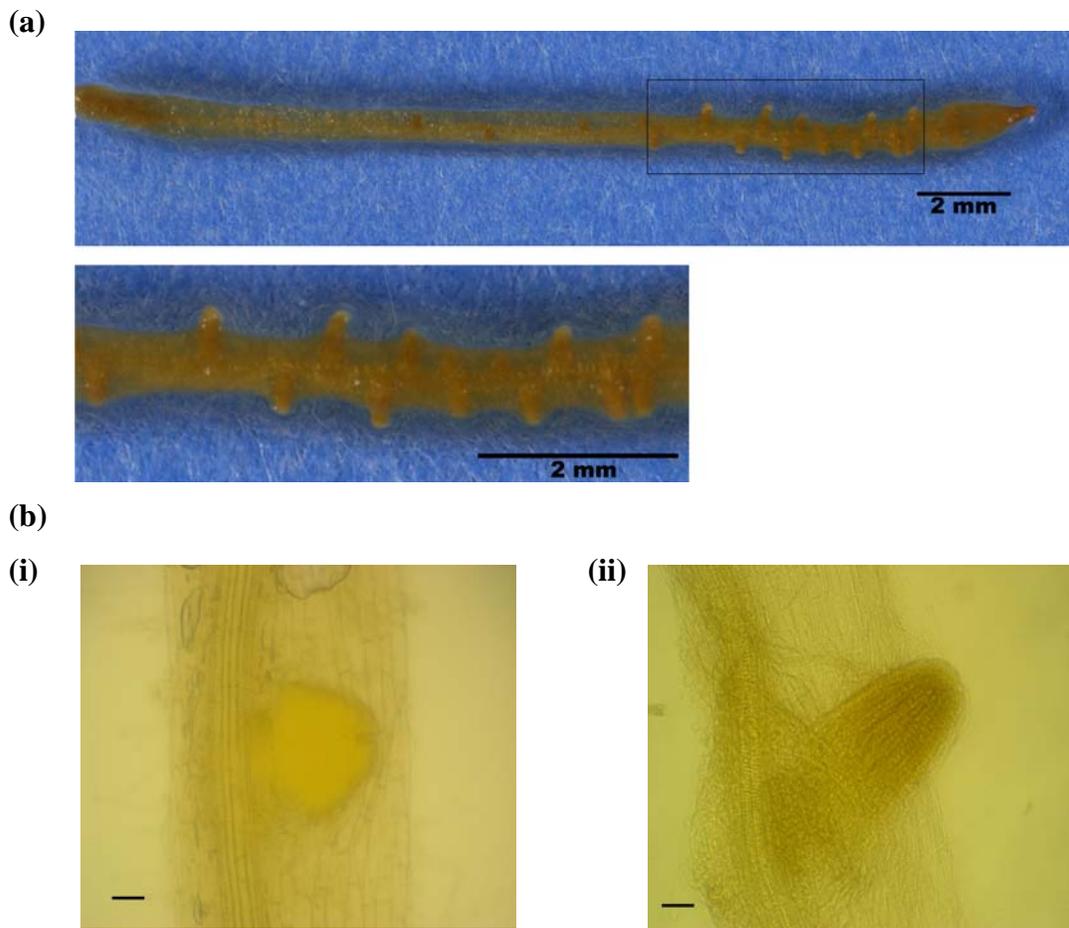


**Plate 3.1:** Morphology of seedlings and an excised root tip grown in White's medium.

- (a) Photograph of 1 cm long seedling root grown in culture media in a growth room with 14 h of light and 10 h of darkness at  $25\pm 1^\circ\text{C}$ .
- (b) Photograph of 2 cm long seedling root grown in culture media in complete darkness at  $25\pm 1^\circ\text{C}$ .
- (c) Photograph of 2 cm long excised root tip grown in culture media in complete darkness at  $25\pm 1^\circ\text{C}$ .

### 3.2 Development of lateral root primordia

LRP developed in 2 cm long excised root tips treated with 1000 nM NAA are shown below. Presence of LRP on the root tip is shown in Plate 3.2 a. Initiation and elongation stages of primordia observed under a light microscope are shown in Plate 3.2 b (i) and (ii) respectively.



#### Plate 3.2: Lateral root primordia

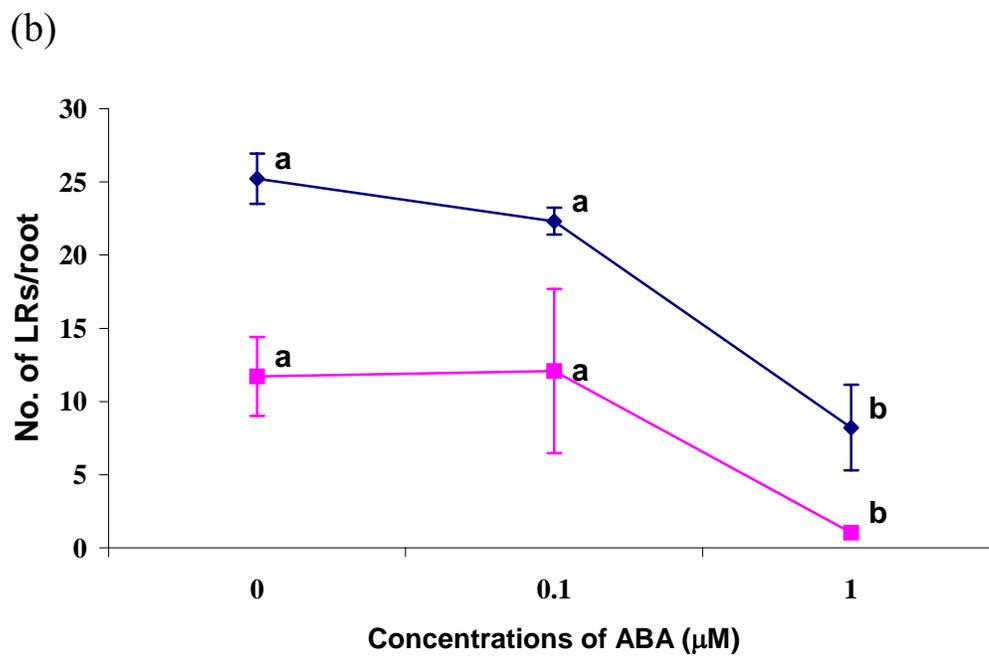
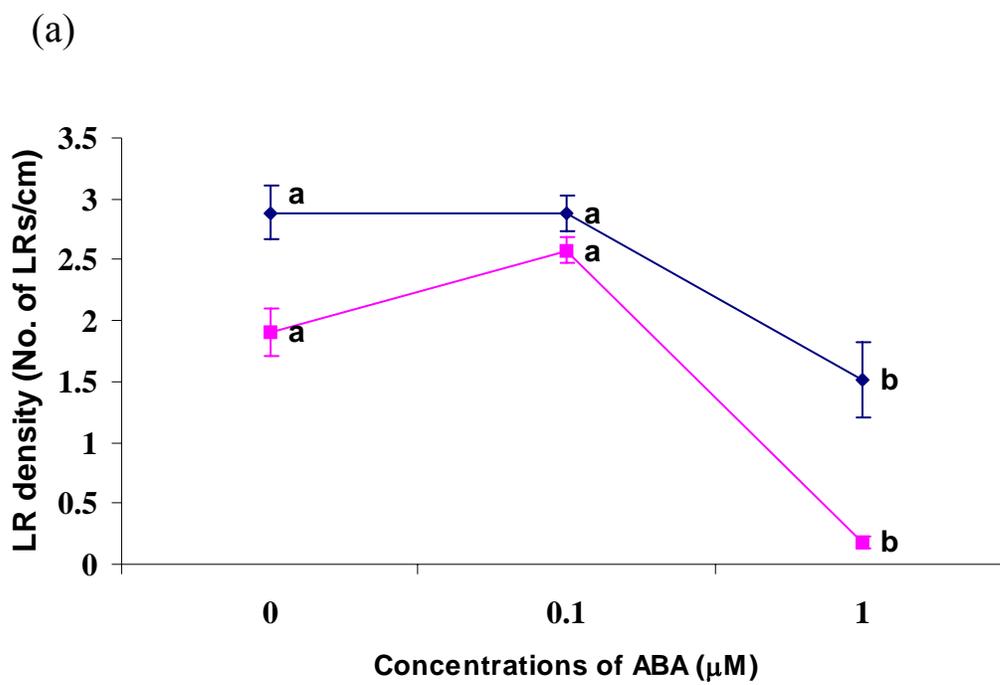
(a) A photograph to describe the position of LRP on a excised root tip treated with 1000 nM NAA was taken with Olympus Camedia 5.1 Mega pixel.

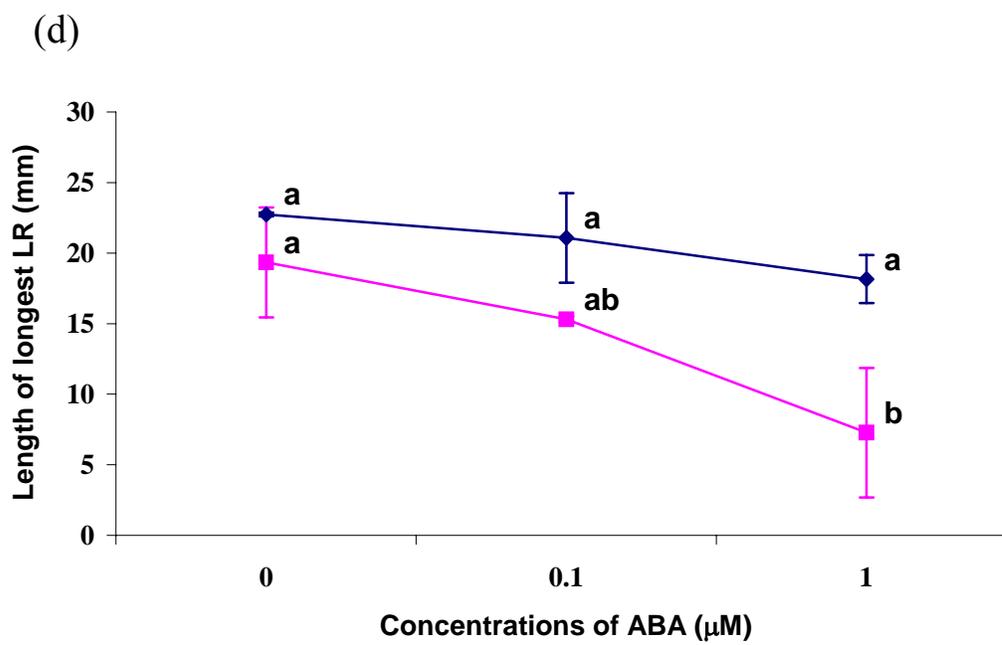
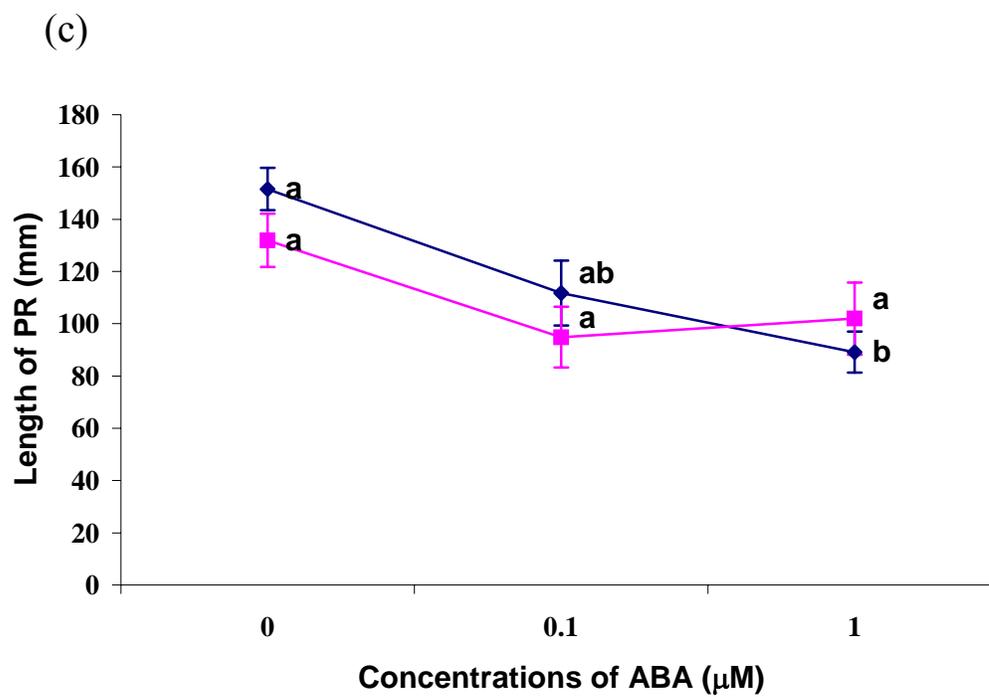
(b) A typical image of a lateral root primordium treated with 5% (W/V) chromium trioxide. Photographs were taken with Olympus Camedia 5.1 Mega pixel under a compound light microscope (Olympus BH2). Primordia development at (i) an early stage (ii) a later stage. Bar = 50 µm

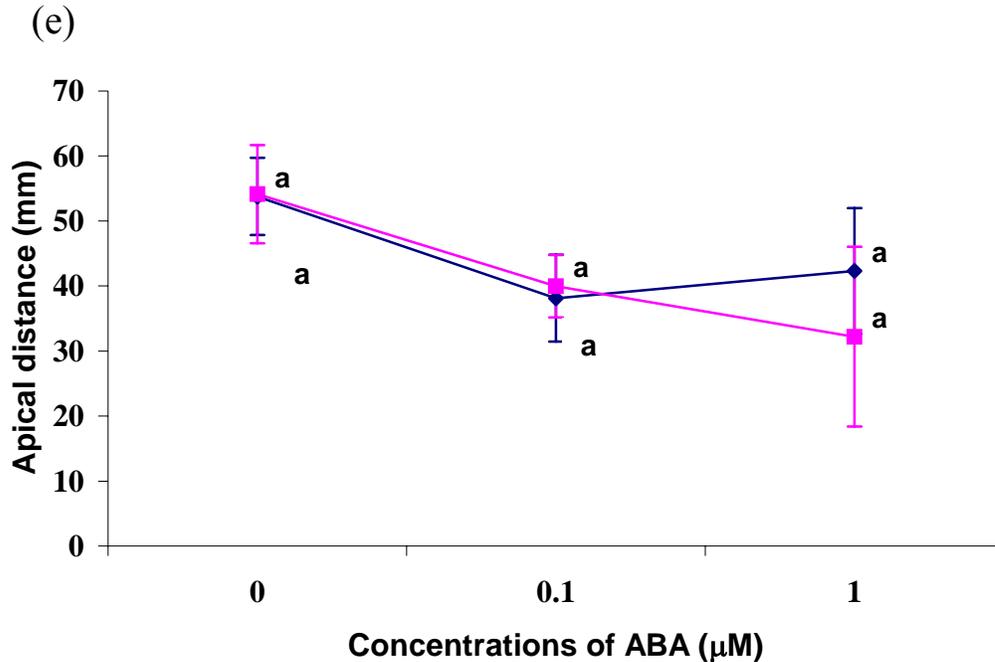
### **3.3 The effect of ABA on seedling roots and excised root tips in complete darkness**

Both seedling roots and excised root tips, which were cultured in complete darkness showed similar responses to the ABA concentrations used in this experiment. The presence of 1  $\mu\text{M}$  ABA in the culture medium significantly reduced the LR density compared with the control in both seedlings roots and the excised root tips (Figure 3.1 a). However, LR density for the excised root tips was less than the seedling roots for the concentrations tested above. Similar responses were observed in the number of LRs (Figure 3.1 b). The PR length of seedling roots was reduced at 1  $\mu\text{M}$  ABA although no statistical difference was observed within the range tested in excised root tips (Figure 3.1 c). The PR length of excised root tip is less than the seedling roots at the same concentrations except at 1  $\mu\text{M}$  ABA, where, it was slightly longer in excised root tips than in seedling roots. The length of the longest LR of excised root tips was reduced at 1  $\mu\text{M}$  ABA. However, no statistical difference was observed within the range tested in seedling roots (Figure 3.1 d). No statistical difference was observed in apical distance of both seedling roots and excised root tips (Figure 3.1 e).

**Figure 3.1** The effects of ABA on 4-day-old, 2 cm long seedling roots and 4-day-old, 2 cm long excised root tips of tomato, cultured at  $25\pm 1^\circ\text{C}$  in complete darkness. **(a)** lateral root density, **(b)** number of lateral roots, **(c)** primary root length, **(d)** length of the longest lateral root and **(e)** apical distance. The values are means  $\pm$  SE from three independent experiments (n=16). Diamonds are used for seedlings and squares are used for excised root tips. Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).





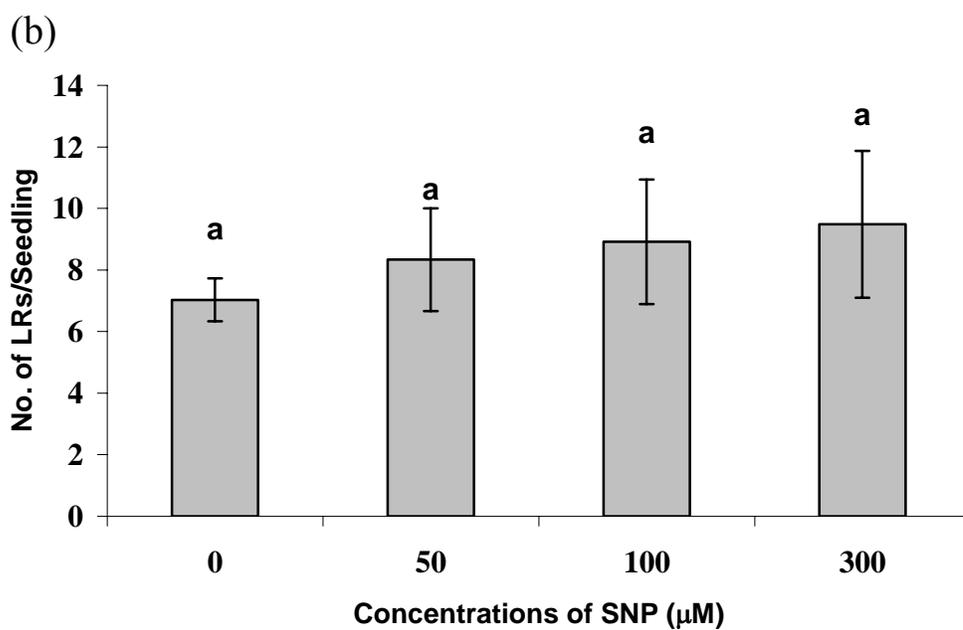
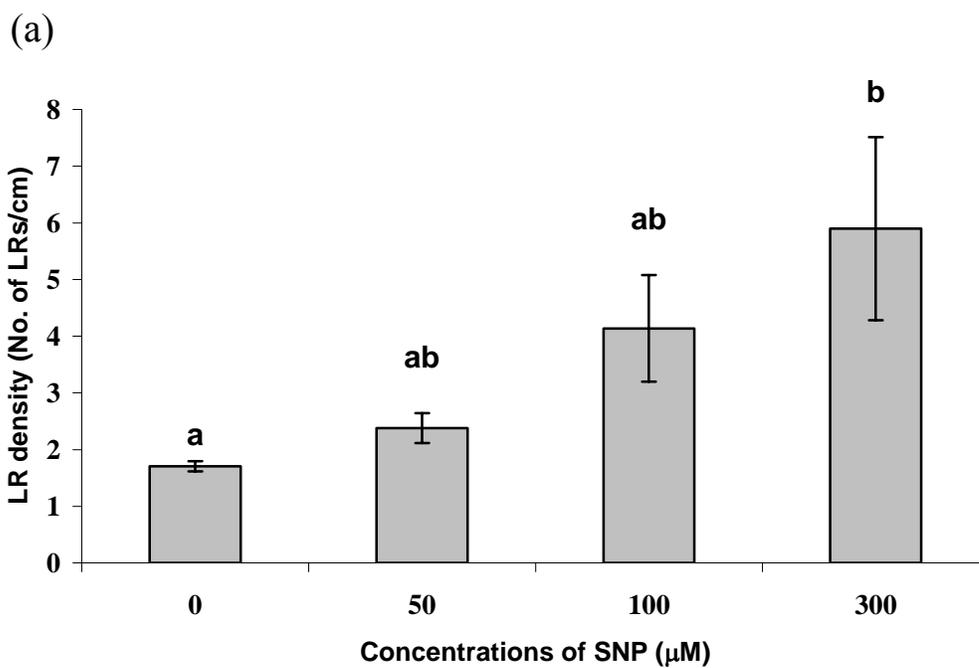


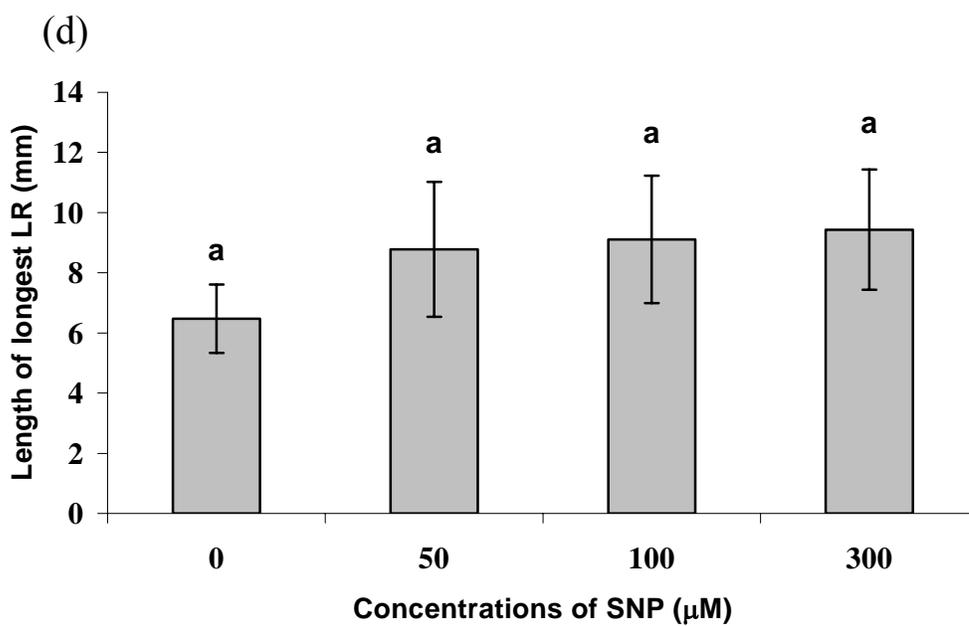
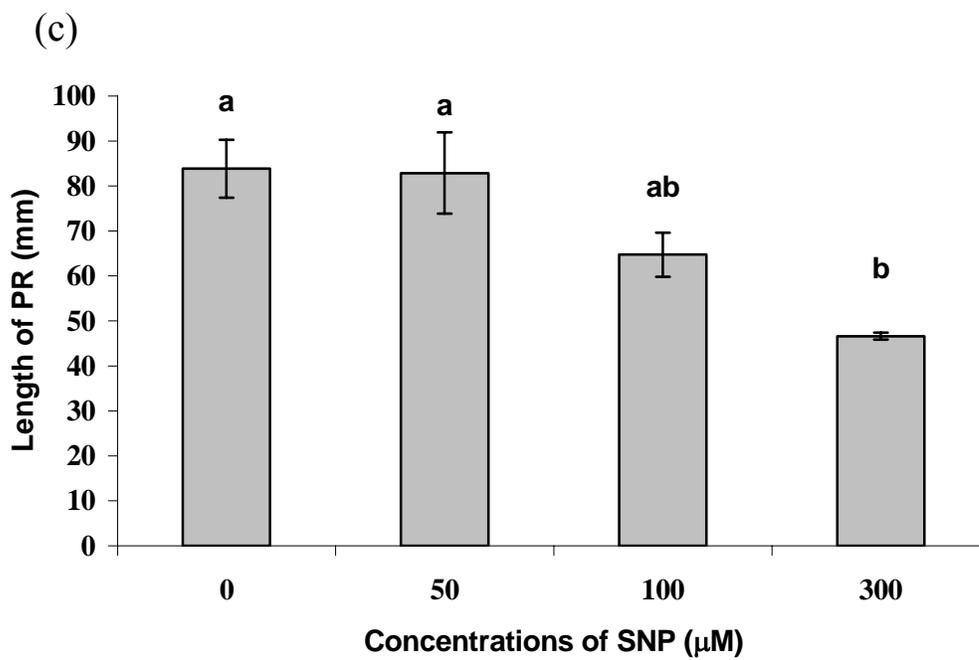
### 3.4 The effect of SNP on seedling roots and excised root tips

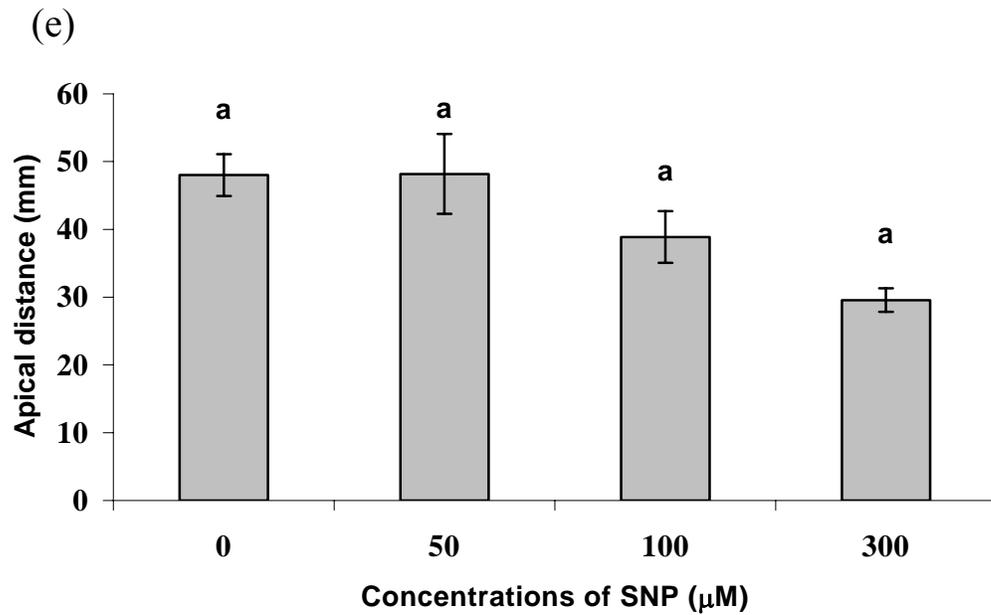
#### 3.4.1 The effect of SNP on seedling roots in a growth room with 14 h of light and 10 h of darkness

A NO donor, SNP, increased LR density in a dose-dependent manner. The presence of 300  $\mu\text{M}$  SNP in the culture medium significantly promoted the LR density compared with the control (Figure 3.2 a). However, no statistical difference was observed in the number of LRs (Figure 3.2 b). In contrast, 300  $\mu\text{M}$  SNP significantly decreased the PR length (Figure 3.2 c). Consequently, opposite patterns were observed with LR density and the PR length (Figures 3.2 a and c). No significant difference was observed in the length of the longest LR and apical distances. However, an increasing trend was observed in the length of the longest LR and a decreasing trend was observed in apical distances (Figures 3.2 d and e).

**Figure 3.2** The effect of SNP on 3-day-old, 1 cm long tomato seedling roots in a growth room with 14 h of light and 10 h of darkness. **(a)** lateral root density, **(b)** number of lateral roots, **(c)** primary root length, **(d)** length of the longest lateral root and **(e)** apical distance. The values are means  $\pm$  SE from 3 independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).







### 3.4.2 The effect of SNP on excised root tips in a growth room with 14 h of light and 10 h of darkness

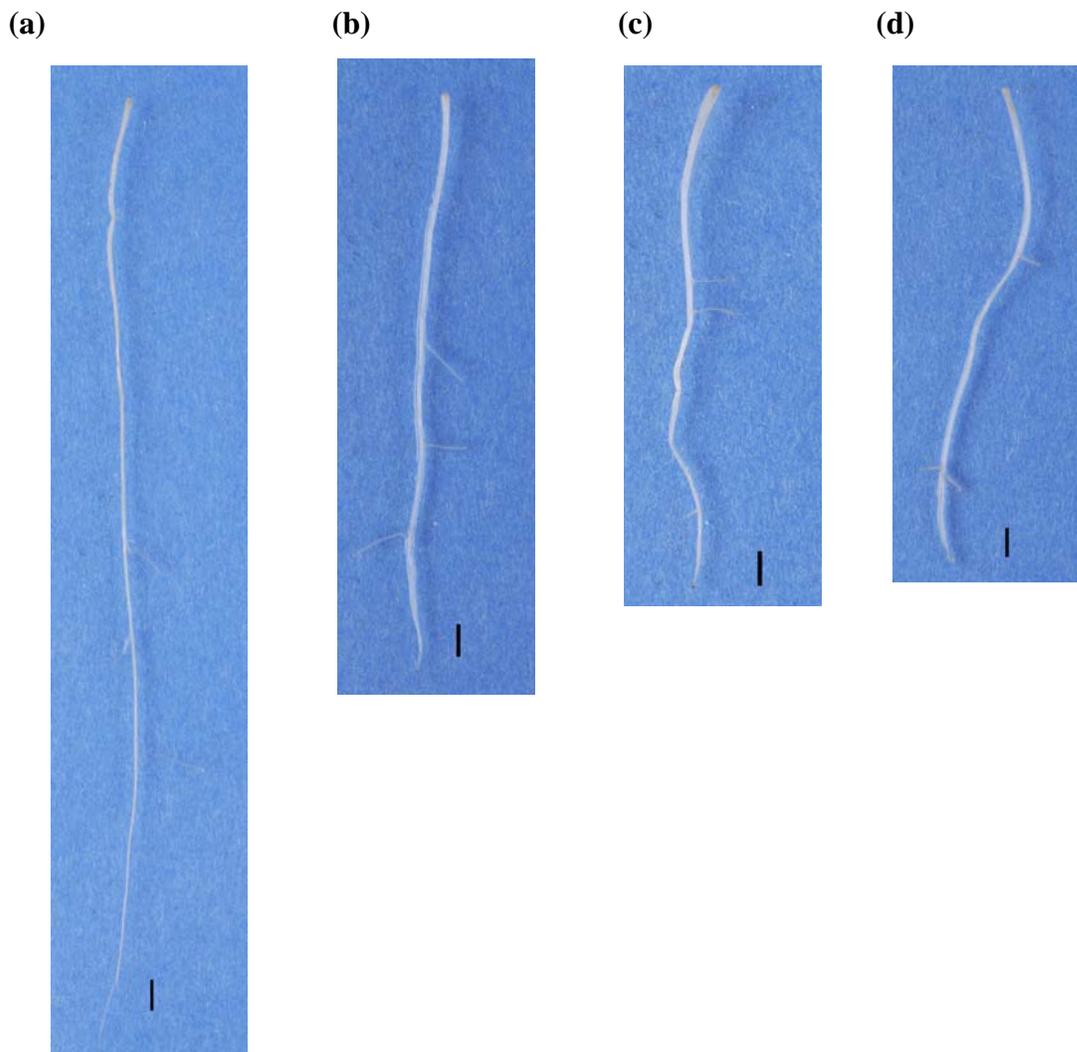
A single 1 cm long root tip was excised from 3-day-old seedlings and cultured in different concentrations of SNP. Compared with the seedling roots, excised root tips responded to the SNP treatments differently. There were no LRs observed in the control. Only a few root tips developed LRs and the number of LRs formed was small in the concentrations of SNP tested. LR development was not observed at the highest concentration tested (500  $\mu$ M). A decreasing trend in PR length was also observed with the increasing concentrations of SNP. A summary of the data is given in Table 3.1.

	LR density (Number of LRs/cm)	Number of LRs/ root	primary root length (mm)	Length of the longest LR (mm)	Apical distance (mm)
<b>Control</b>	0	0	36.80 $\pm$ 4.92	0	0
<b>10 <math>\mu</math>M SNP</b>	0.18 $\pm$ 0.08	0.49 $\pm$ 0.17	48.45 $\pm$ 1.14	1.04 $\pm$ 0.74	17.22 $\pm$ 6.70
<b>50 <math>\mu</math>M SNP</b>	0.13 $\pm$ 0.04	0.23 $\pm$ 0.03	34.64 $\pm$ 1.45	0.37 $\pm$ 0.06	4.57 $\pm$ 0.03
<b>200 <math>\mu</math>M SNP</b>	0.09 $\pm$ 0.02	0.16 $\pm$ 0.03	19.47 $\pm$ 0.59	0.22 $\pm$ 0.04	2.18 $\pm$ 0.17
<b>300 <math>\mu</math>M SNP</b>	0.50 $\pm$ 0.20	0.56 $\pm$ 0.01	17.07 $\pm$ 0.92	0.72 $\pm$ 0.31	3.46 $\pm$ 1.25
<b>500 <math>\mu</math>M SNP</b>	0	0	15.85 $\pm$ 0.48	0	0

**Table 3.1** The effects of SNP on 3-day-old, 1 cm long excised root tips of tomato incubated in a growth room with 14 h of light and 10 h of darkness. The values are means  $\pm$  SE from 3 independent experiments (n=16).

### 3.4.3 The effect of SNP on excised root tips in complete darkness

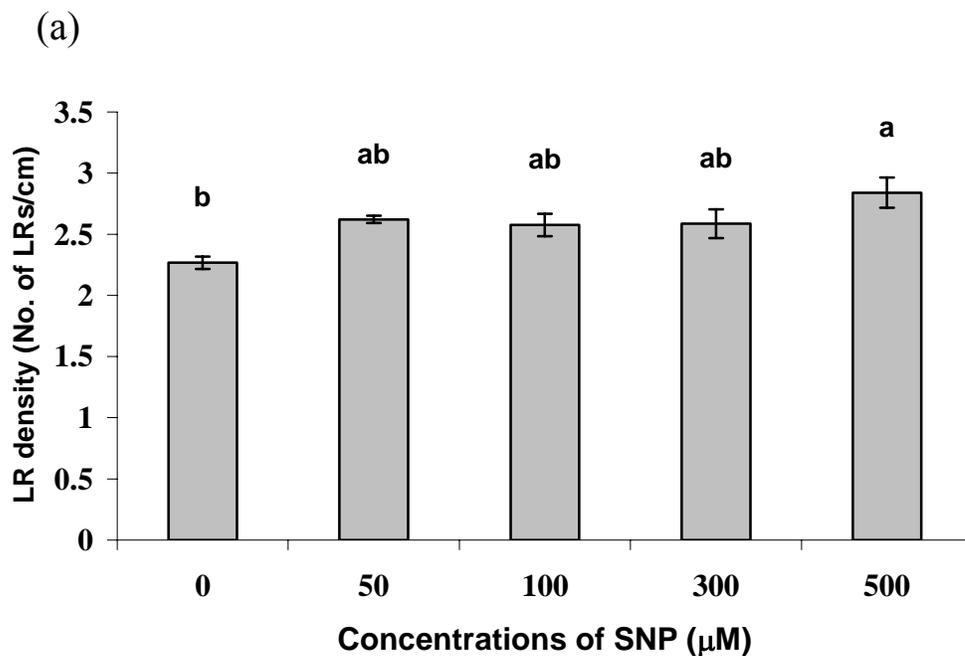
The same experiment (as described in Section 3.4.2) was carried out except that 2 cm long excised root tips were incubated in complete darkness at  $25\pm 1^\circ\text{C}$ . Excised root tips treated with different concentrations of SNP are shown in Plate 3.3. The PR of SNP treated root tips were thicker and shorter than the control.

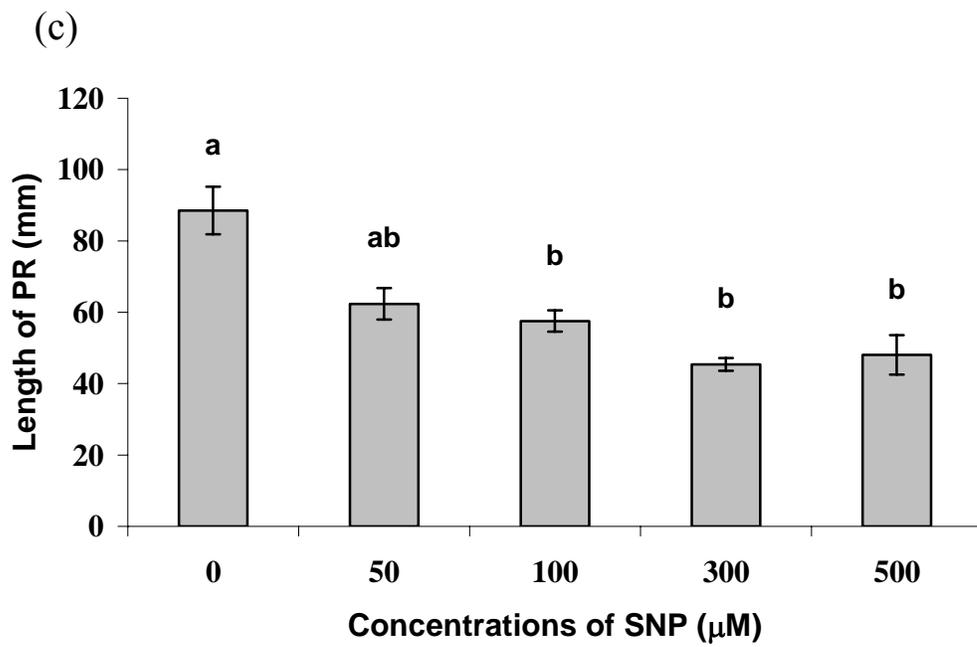
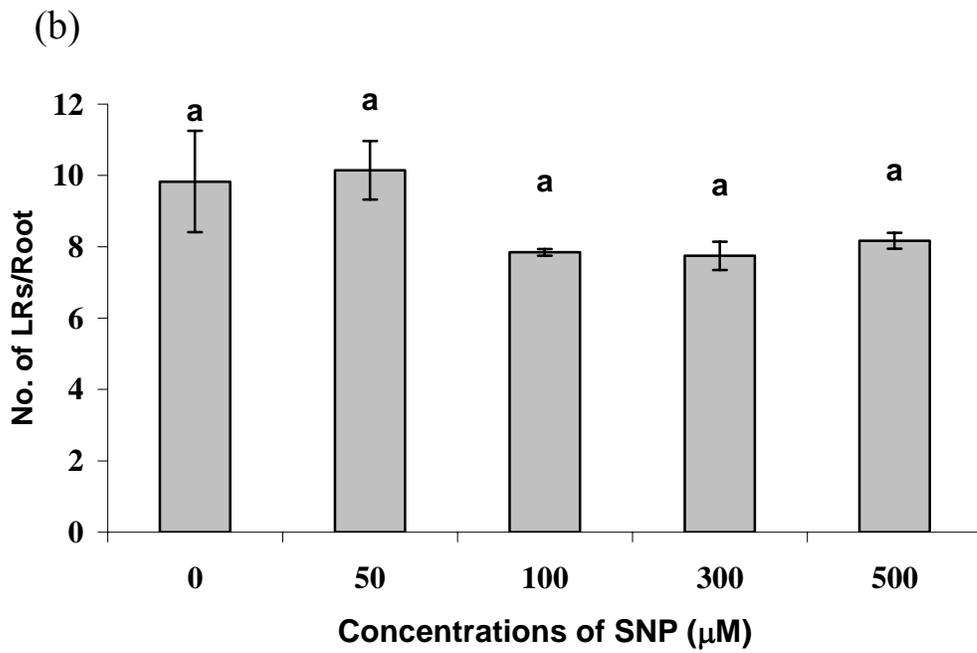


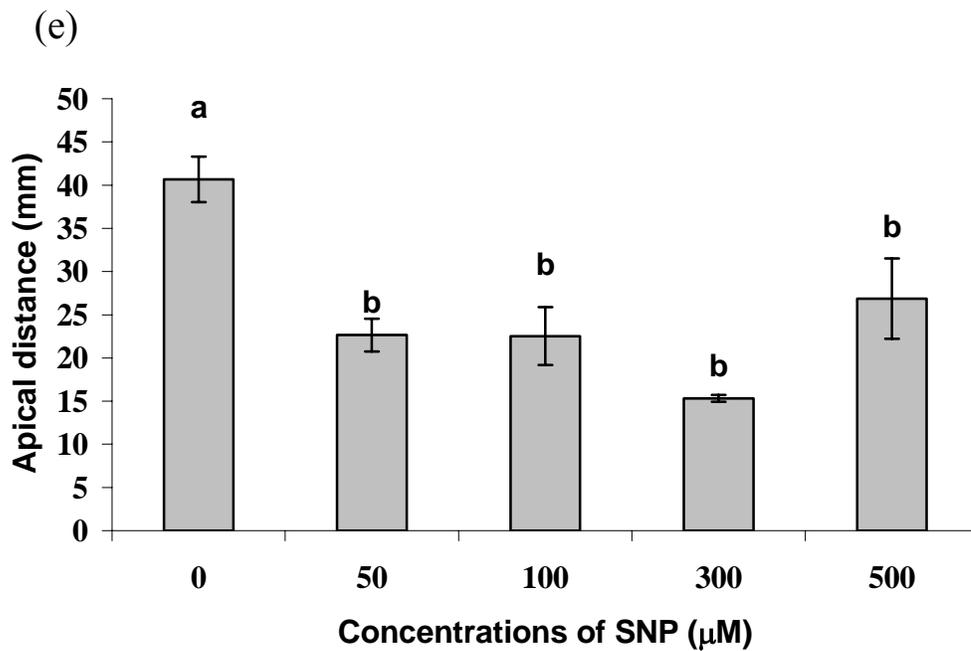
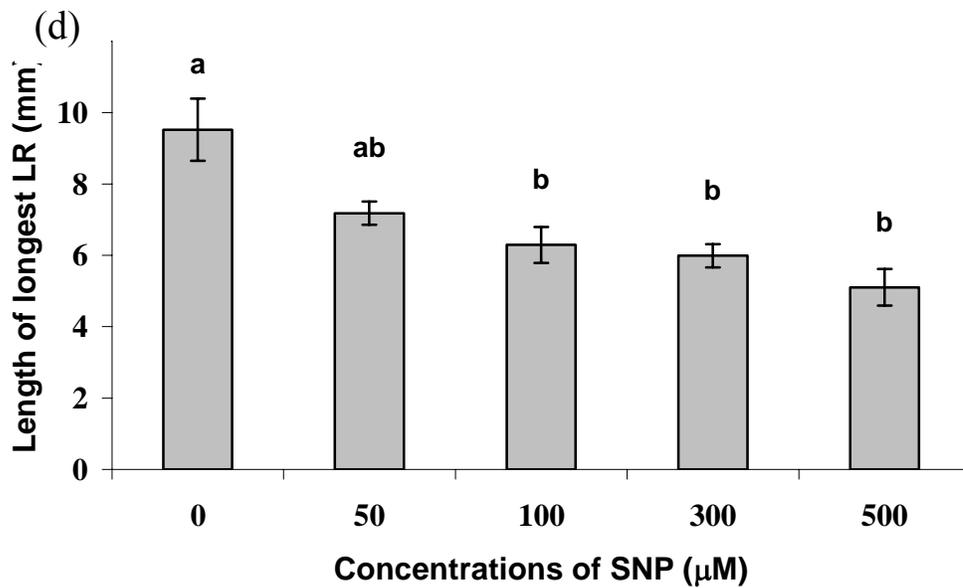
**Plate 3.3** Photographs of 2 cm long excised root tips of tomato cultured for 7 days in complete darkness at  $25\pm 1^\circ\text{C}$ . Root tips were treated with (a) 0  $\mu\text{M}$  SNP (the control), (b) 50  $\mu\text{M}$  SNP, (c) 100  $\mu\text{M}$  SNP and (d) 300  $\mu\text{M}$  SNP. Bar = 2 mm

**Figure 3.3** The effects of SNP on 4-day-old, 2 cm long excised root tips of tomato cultured in complete darkness. **(a)** lateral root density, **(b)** number of lateral roots, **(c)** primary root length, **(d)** length of the longest lateral root and **(e)** apical distance. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

It was found that 500  $\mu\text{M}$  SNP in the culture medium significantly promoted the LR density compared with the control (Figure 3.3 a). No statistical difference was observed in the number of LRs within the concentrations tested above (Figure 3.3 b), but a decreasing trend was observed. 100  $\mu\text{M}$  or higher concentrations of SNP significantly reduced the PR length and the length of the longest LR (Figures 3.3 c and d) while 50  $\mu\text{M}$  or higher concentrations of SNP significantly reduced the apical distances (Figure 3.3 e)







### 3.5 Interaction between ABA and SNP on excised root tips of tomato

In order to evaluate the interaction between ABA and SNP, different concentrations of SNP were mixed with 1  $\mu\text{M}$  ABA. Treatments with 500 or 1000  $\mu\text{M}$  SNP led to significant increase in the LR density with respect to the control (a basal medium without any plant growth regulators), although a decreasing trend of the stimulatory effect of these three SNP treatments was observed (Figure 3.4). Compared with the control, 1  $\mu\text{M}$  ABA reduced the LR density. The combination of 1  $\mu\text{M}$  ABA with 500, 1000, 1400 or 2000  $\mu\text{M}$  SNP showed significantly increased LR density compared to ABA alone. However, 1  $\mu\text{M}$  ABA with 2000  $\mu\text{M}$  SNP showed significantly reduced LR density than the other concentrations. One  $\mu\text{M}$  ABA added to 500 or 1000  $\mu\text{M}$  SNP significantly decreased LR density compared with the respective SNP concentrations alone, but 1  $\mu\text{M}$  ABA added to 1400 or 2000  $\mu\text{M}$  SNP did not significantly decreased LR density compared with the respective SNP concentrations alone.

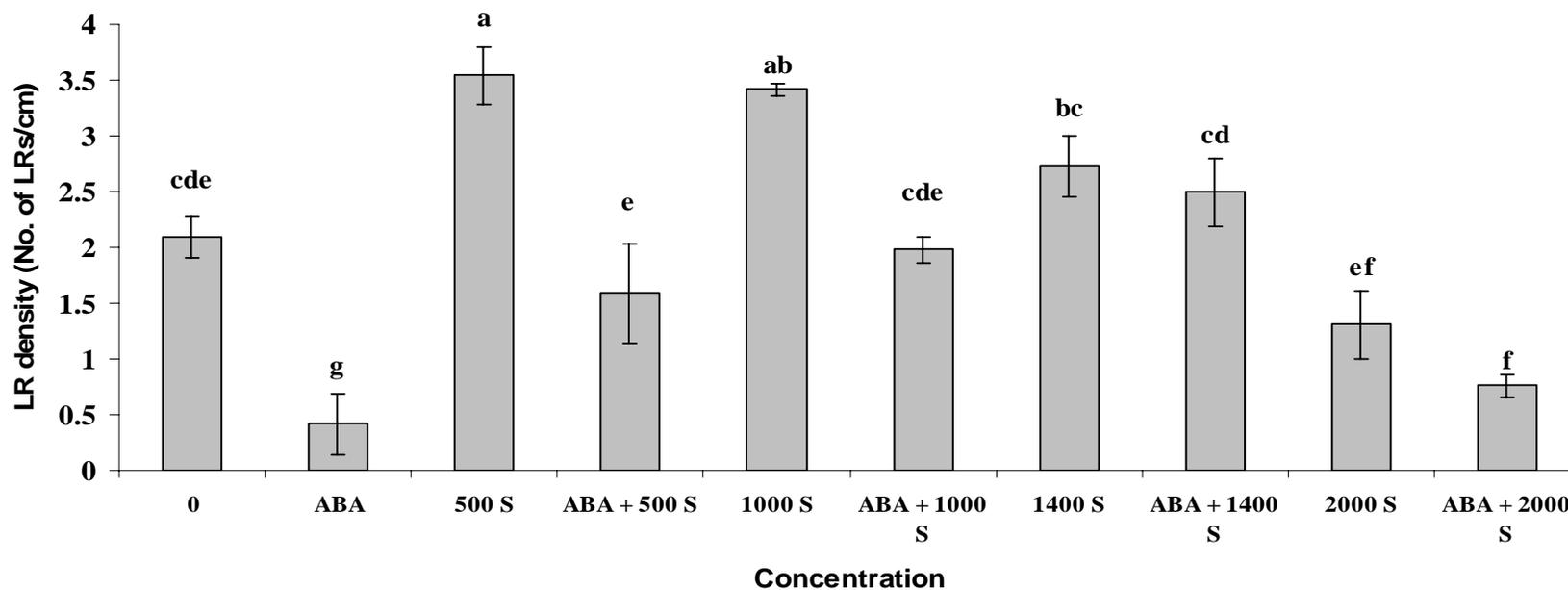
A similar number of LRs was observed in 500  $\mu\text{M}$  SNP compared with the control. However, a statistically significant decrease was observed in the number of LRs (Figure 3.5) as a function of the increasing concentrations of SNP (500, 1000, 1400  $\mu\text{M}$  and 2000  $\mu\text{M}$  SNP). The combination of 1  $\mu\text{M}$  ABA with 500 or 1000  $\mu\text{M}$  SNP resulted in an increasing trend in the number of LRs compared with 1  $\mu\text{M}$  ABA alone.

There was no significant difference between the control and 1  $\mu\text{M}$  ABA as far as PR length was concerned (Figure 3.6). All other treatments resulted in significantly shorter PR compared with the control.

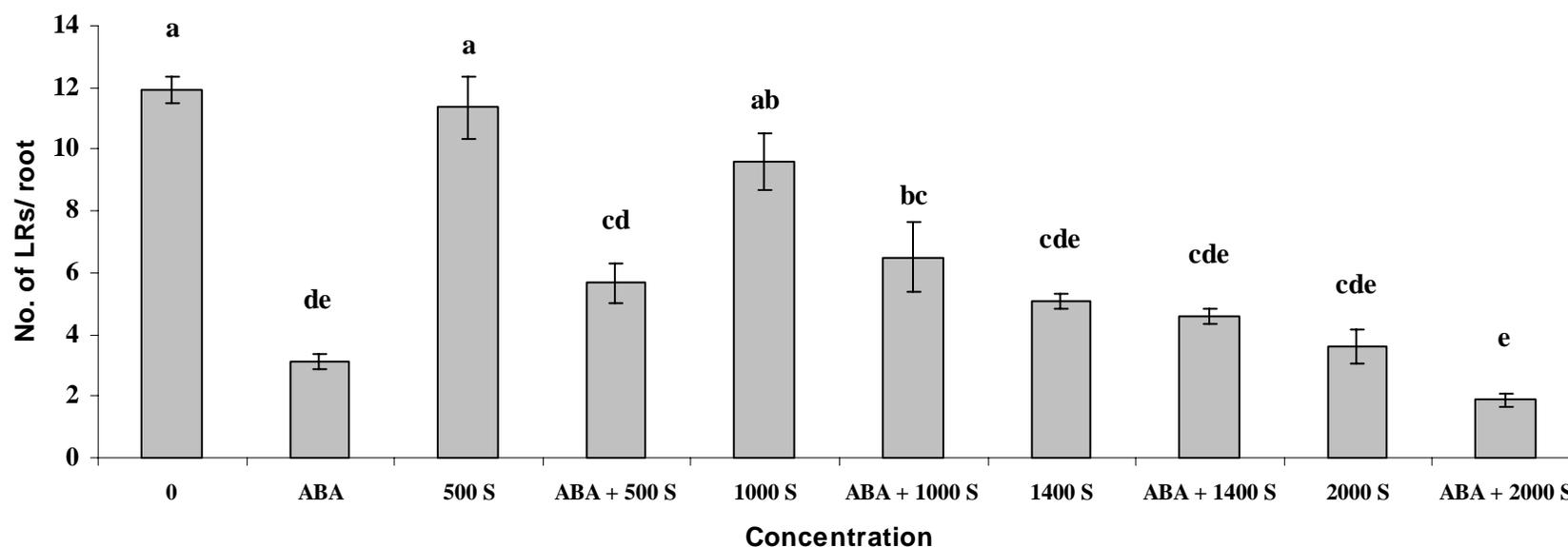
There was no significant difference among the control, 1  $\mu\text{M}$  ABA and 500  $\mu\text{M}$  SNP in the length of the longest LR (Figure 3.7). Also there was no statistically significant difference among the different concentrations of SNP tested (500, 1000, 1400 and 2000  $\mu\text{M}$  SNP). In addition, the combination of 1  $\mu\text{M}$  ABA with 500  $\mu\text{M}$  or higher concentrations of SNP did not show any significant difference in the length of the longest LR compared with the respective SNP concentrations alone.

As in the PR length, no significant difference was observed between the control and 1  $\mu\text{M}$  ABA on apical distances (Figure 3.8). All the other treatments resulted in reduced apical distances and there was no significant difference among them.

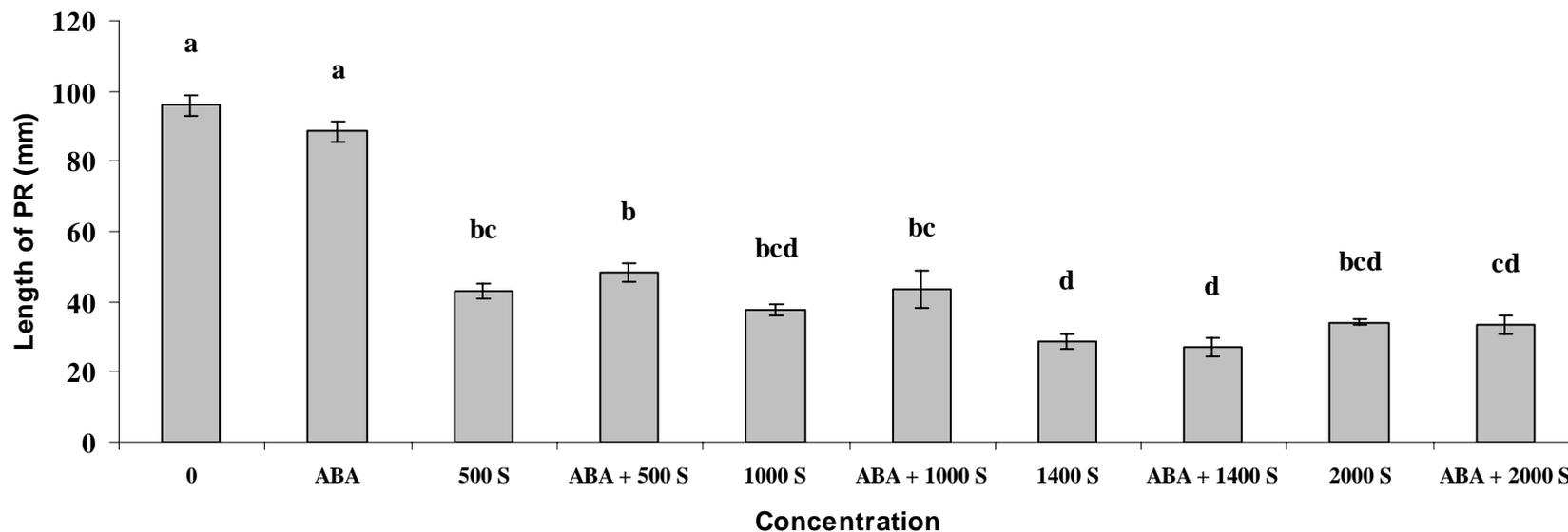
Except from the preliminary experiments with either ABA or SNP, the experiment to find the relationship between ABA and SNP was carried out with the same variety of tomato seeds, but purchased from a different company, Asian Seeds. Some of the effective concentrations (0, 300, 500  $\mu\text{M}$  SNP) were repeated and confirmed that the new seeds are behaving the same as the previous seeds.



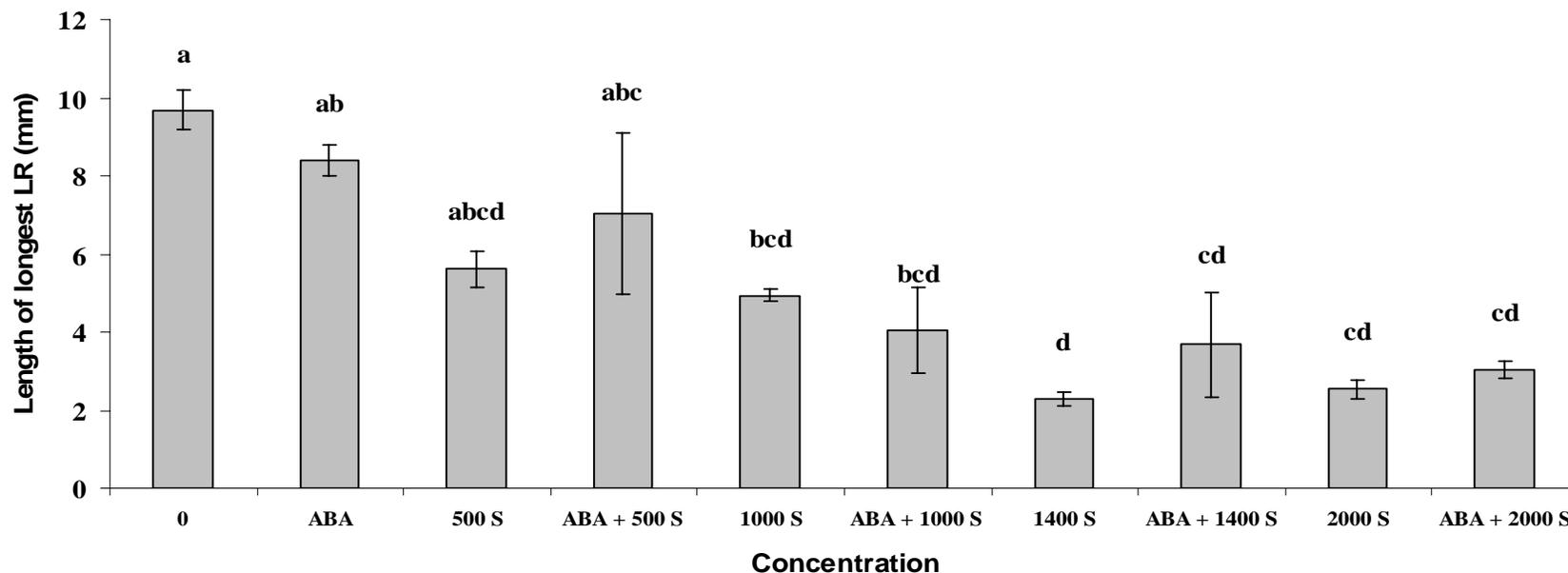
**Figure 3.4** Interaction between ABA and SNP on LR density. The effect of 1  $\mu\text{M}$  ABA (ABA) and different concentrations of SNP ( $\mu\text{M}$ ) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $p < 0.05$  (t-test).



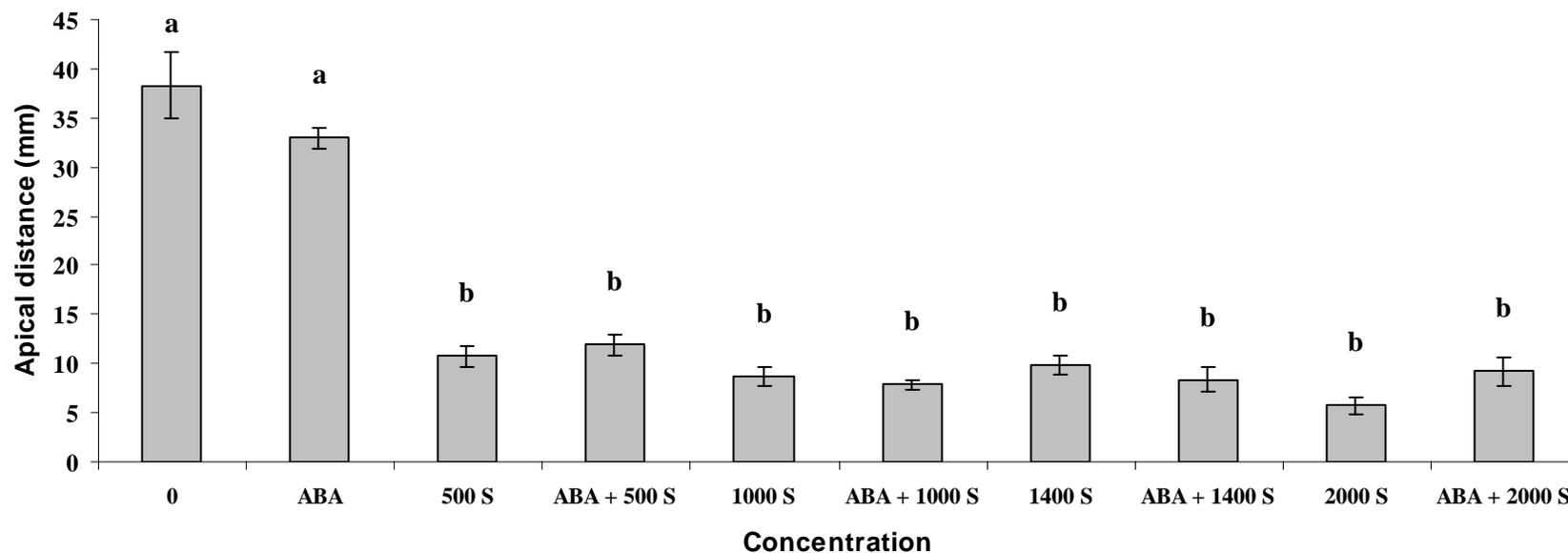
**Figure 3.5:** Interaction between ABA and SNP on the number of LRs. The effect of 1  $\mu\text{M}$  ABA (ABA) and different concentrations of SNP ( $\mu\text{M}$ ) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $p<0.05$  (t-test).



**Figure 3.6:** Interaction between ABA and SNP on the PR length. The effect of 1  $\mu\text{M}$  ABA (ABA) and different concentrations of SNP ( $\mu\text{M}$ ) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $p < 0.05$  (t-test).



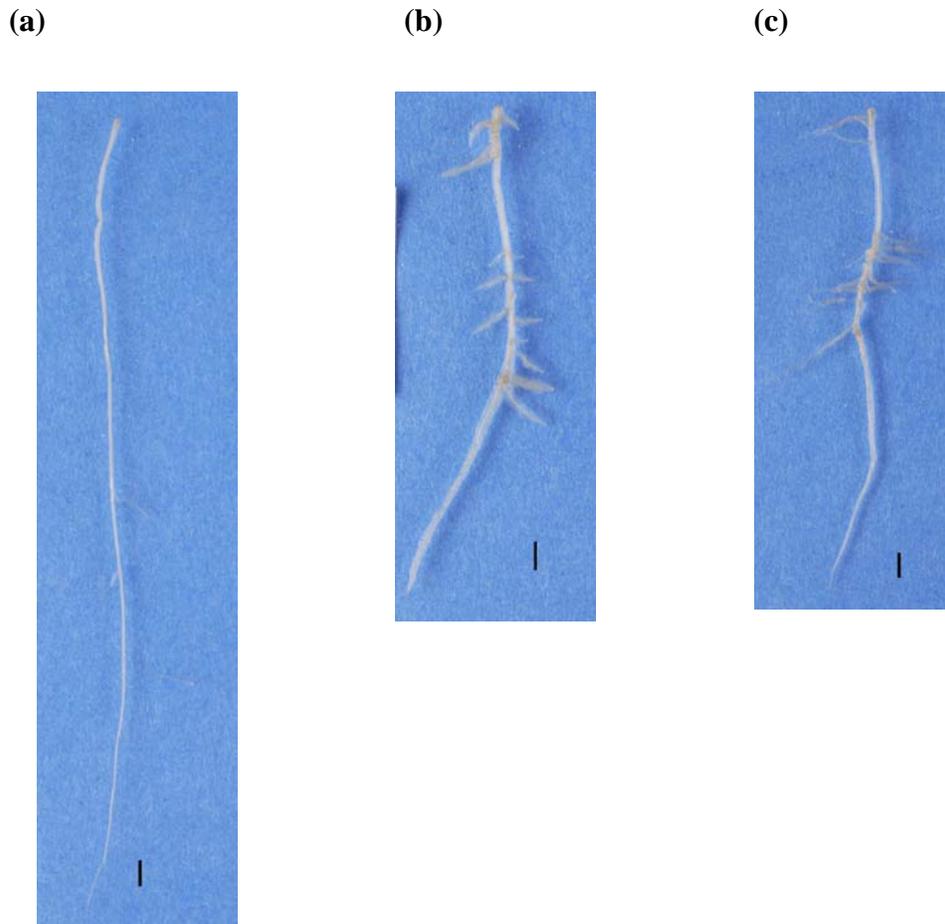
**Figure 3.7:** Interaction between ABA and SNP on the length of the longest LR. The effect of 1  $\mu$ M ABA (ABA) and different concentrations of SNP ( $\mu$ M) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $p < 0.05$  (t-test).



**Figure 3.8:** Interaction between ABA and SNP on apical distance. The effect of 1  $\mu$ M ABA (ABA) and different concentrations of SNP ( $\mu$ M) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $n<0.05$  (t-test).

### 3.6 The effect of NAA on excised root tips of tomato

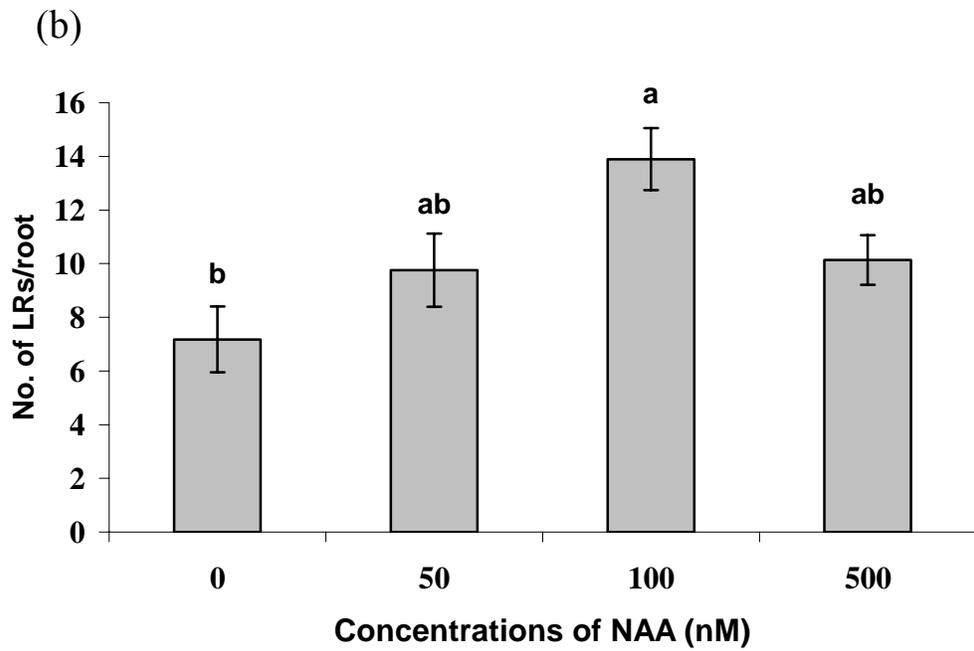
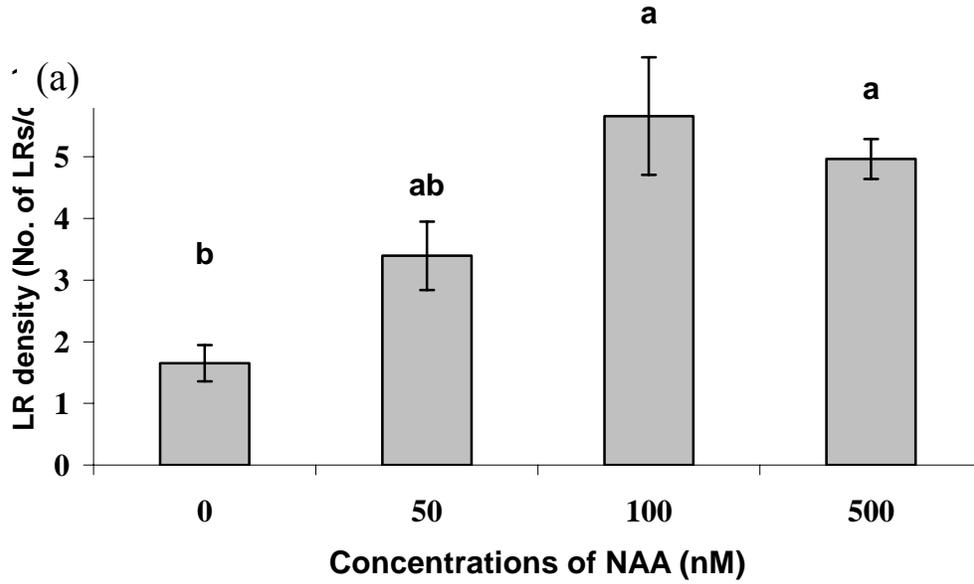
Root tips treated with different concentrations of NAA are shown in plate 3.4. The PR of NAA treated root tips were thicker and shorter than the control.

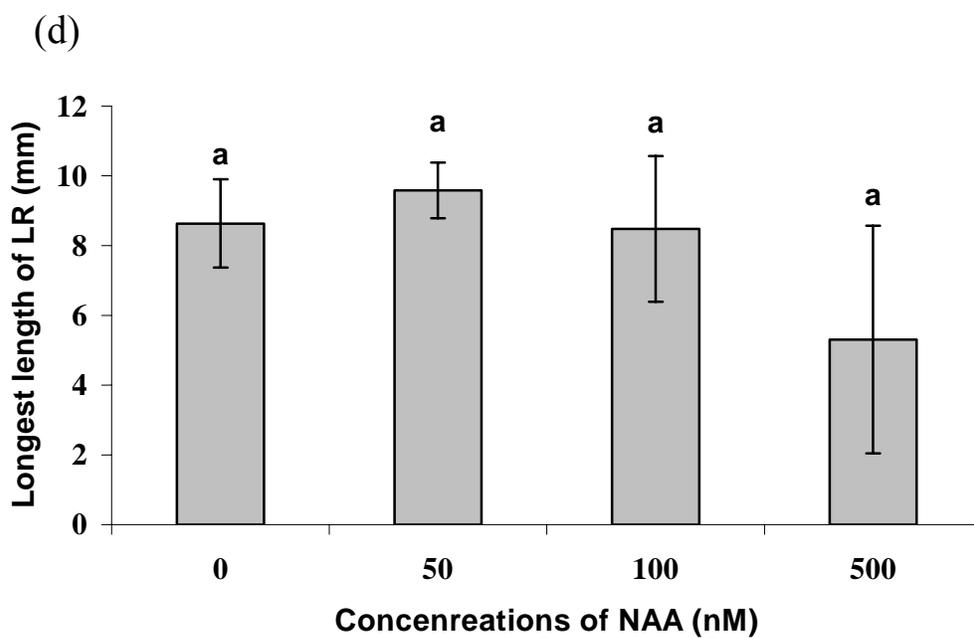
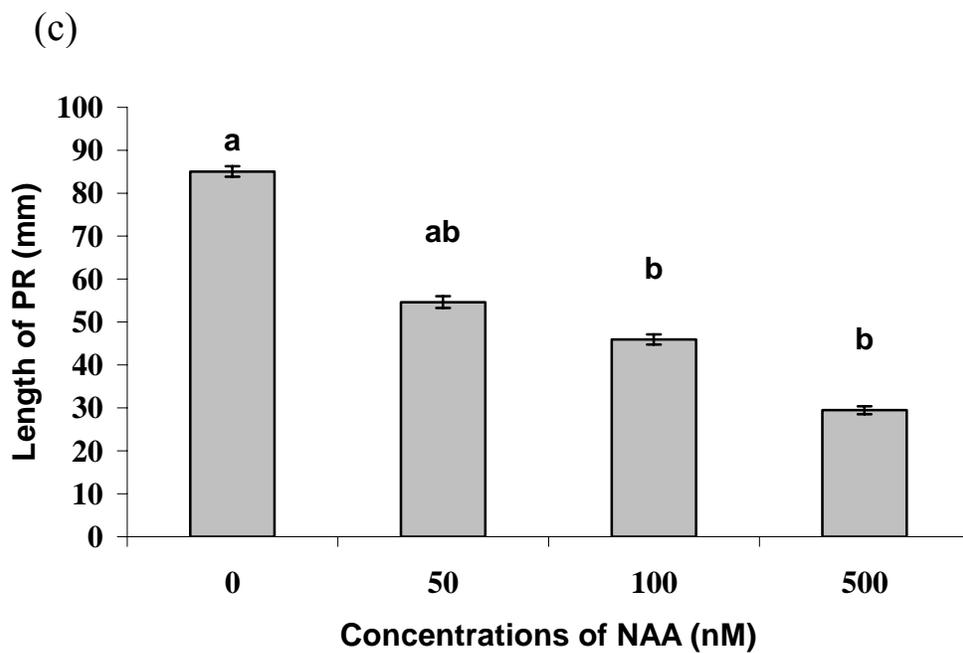


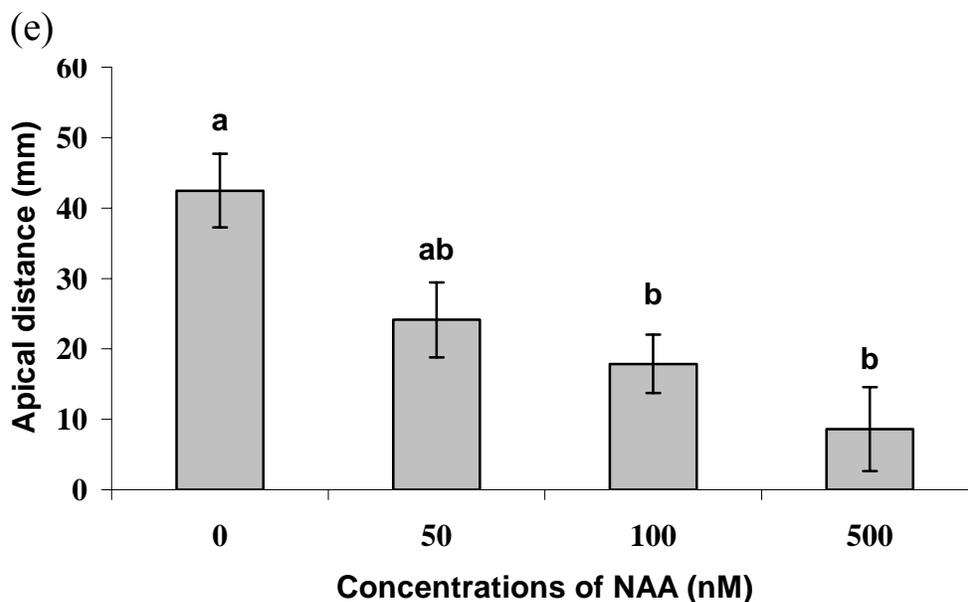
**Plate 3.4** Photographs of 2 cm long excised root tips of tomato incubated for 7 days in different concentrations of NAA in darkness at  $25\pm 1^\circ\text{C}$ . Treatment of root tips with (a) 0 nM NAA, (b) 50 nM NAA, (c) 100 nM NAA and. Bar = 2 mm

**Figure 3.9** The effect of NAA on 4-day-old, 2 cm long excised root tips of tomato. **(a)** lateral root density, **(b)** number of lateral roots, **(c)** primary root length, **(d)** length of the longest lateral root and **(e)** apical distance. The values are means  $\pm$  SE from four independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

There was a dose-dependent increase in LR density and the number of LRs in response to the NAA treatments. It was found that 100 nM NAA significantly promoted the LR density (Figure 3.9 a). A decreasing trend was observed at 500 nM compared with 100 nM, even though there was no statistical difference observed (Figure 3.9 a). A similar effect of NAA on the number of LRs was also observed (Figure 3.9 b). The PR length and apical distances were reduced in response to the NAA treatments in a dose-dependent manner (Figures 3.9 c and e). It was found that 100 or 500 nM NAA resulted in a significant decrease in the PR length compared with the control (Figure 3.9 c). However, no statistical difference was observed in the length of the longest LR (Figure 3.9 d) among the concentrations of NAA tested.







### 3.7 The effect of fluridone on excised root tips of tomato

The effect of fluridone, an ABA biosynthesis inhibitor, on excised root tips of tomato is shown in Figure 3.10. There was no statistical difference observed in the LR density among the concentrations of the fluridone tested (Figure 3.10 a (i)). However, 1  $\mu$ M fluridone significantly increased primordia density and therefore, LR plus primordia density (Figure 3.10 a (ii) and (iii)). The same pattern of response was observed in the number of LRs or primordia (Figure 3.10 b). However, there was no significant difference observed in the PR length or the length of the longest LRs or apical distances in response to the concentrations of fluridone tested (Figures 3.10 c to e).

**Figure 3.10** The effect of fluridone on 4-day-old, 2 cm long tomato excised root tips. **(a) (i)**

lateral root density

**(a) (ii)** Primordia density

**(a) (iii)** Lateral plus primordia density

**(b) (i)** number of lateral roots

**(b) (ii)** number of primordia

**(b) (iii)** number of lateral plus primordia

**(c)** primary root length

**(d)** length of the longest lateral root and

**(e)(i)** lateral root apical distance.

**(e)(ii)** primordia apical distance

**(e)(iii)** lateral root or primordia apical distance (distance to either first LR or primordia from the root rip)

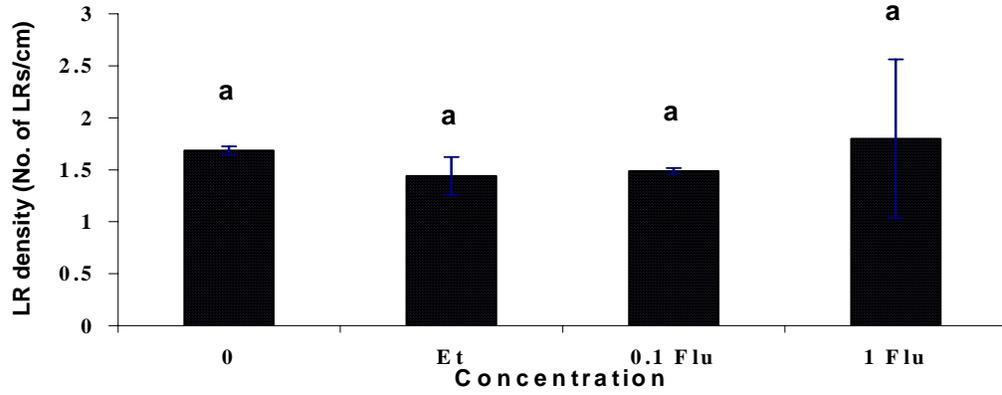
Et – 100 % ethanol

0.1 Flu – 0.1  $\mu$ M fluridone

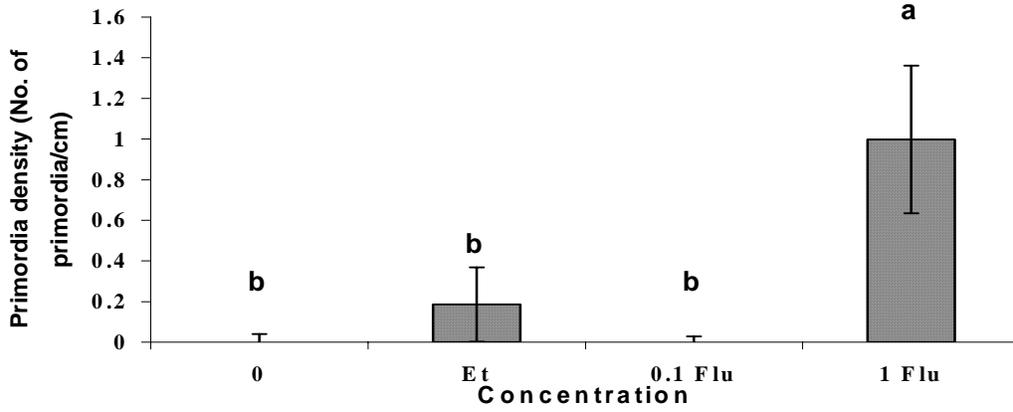
1 Flu – 1  $\mu$ M fluridone

The values are means  $\pm$  SE from 3 independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

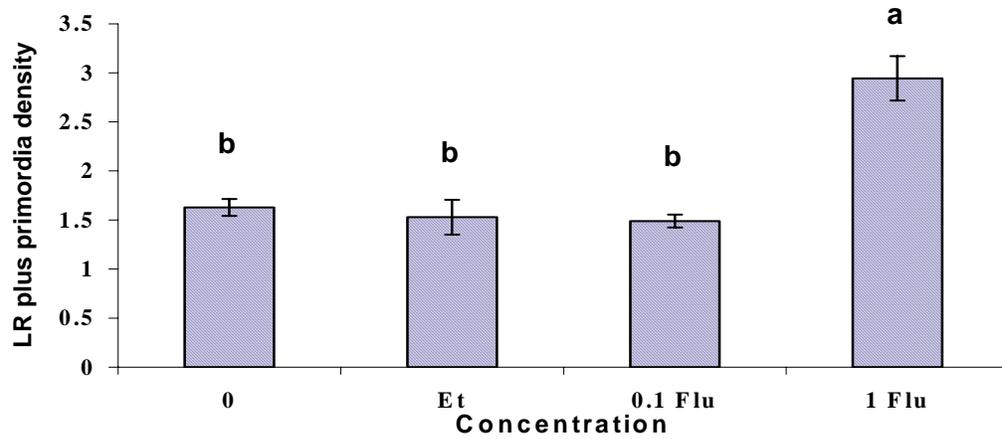
(a) (i)



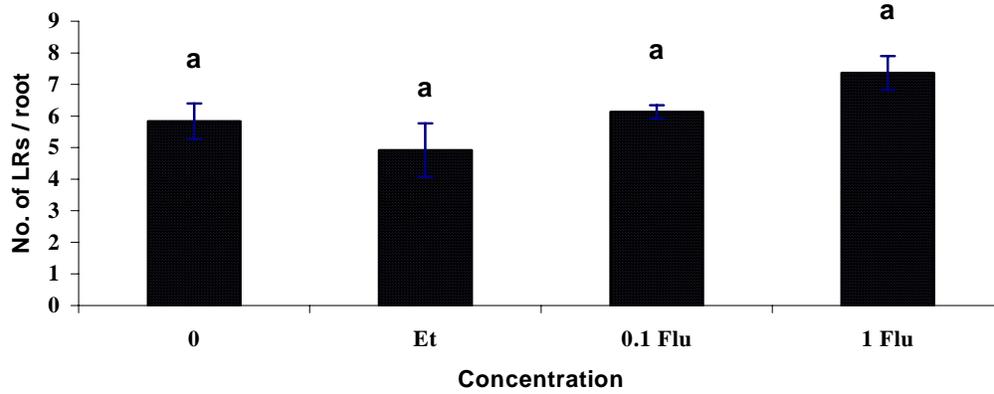
(ii)



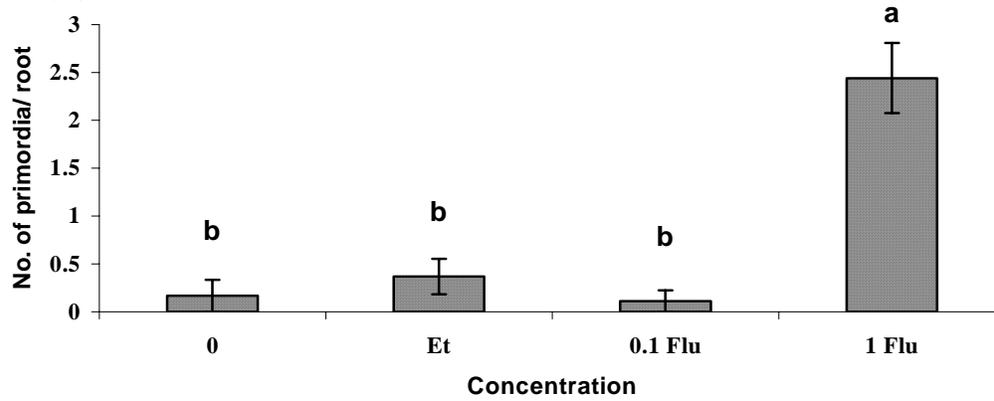
(iii)



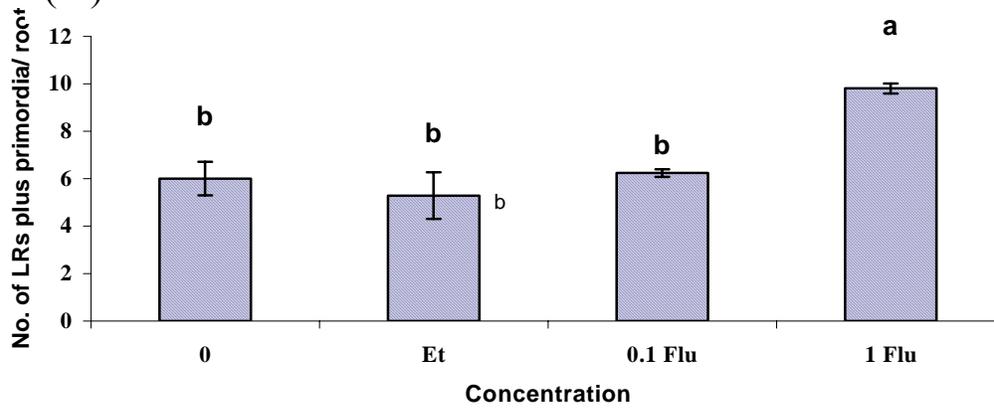
(b) (i)

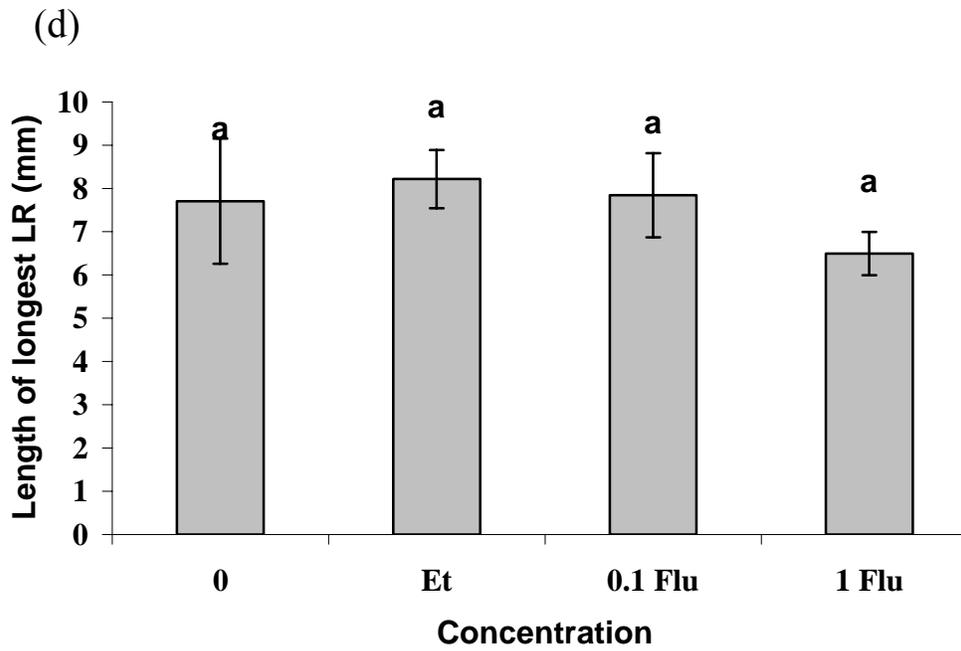
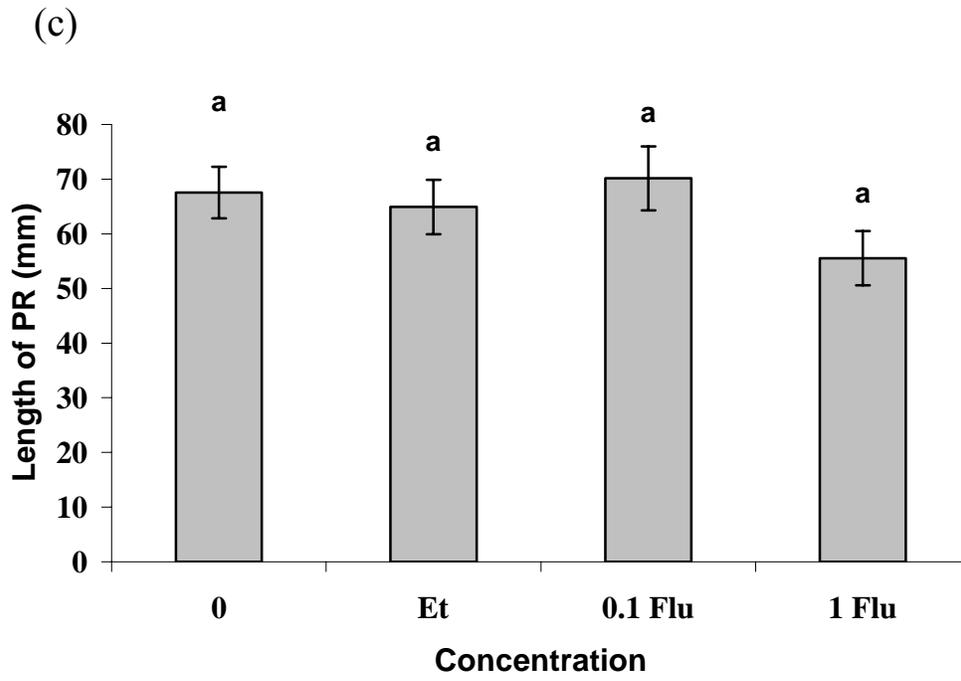


(ii)

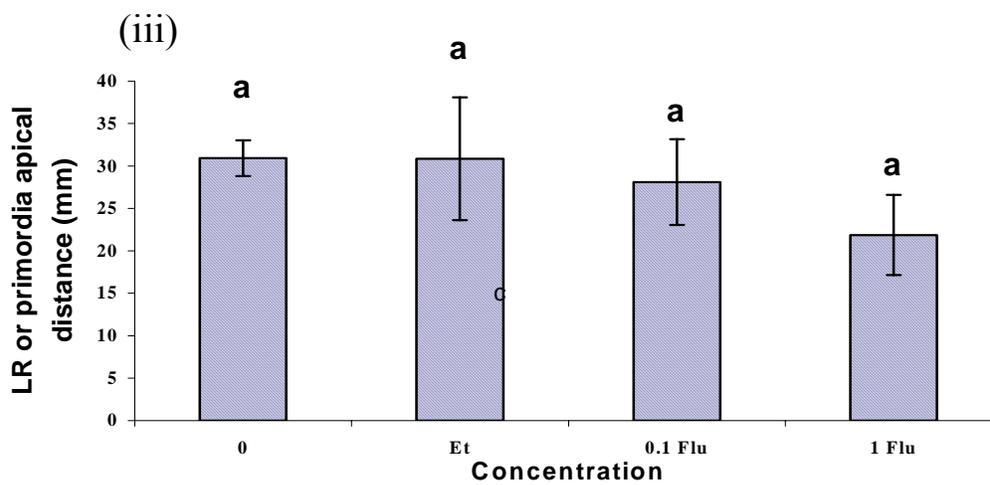
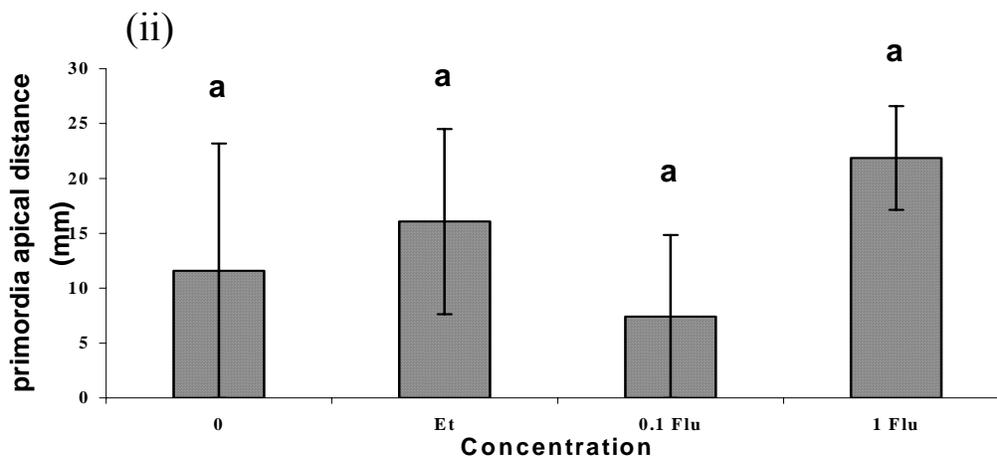
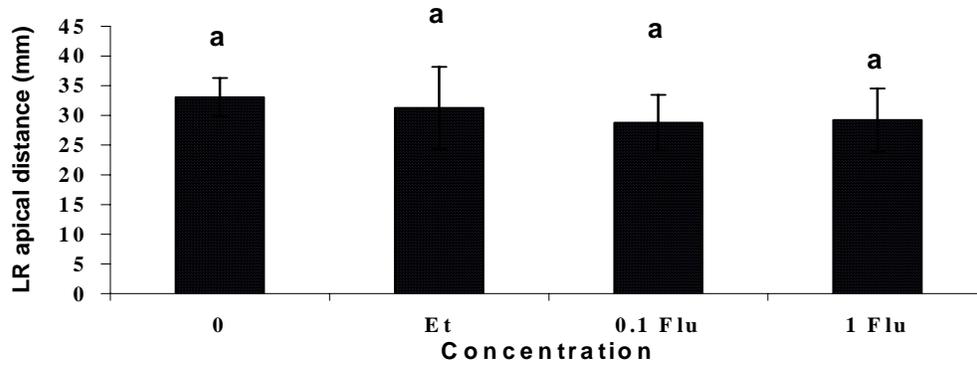


(iii)





(e) (i)



### 3.8 Interaction between NAA and SNP on excised root tips of tomato

In order to evaluate the interaction between NAA and SNP, the effect of various concentrations of NAA (50, 100, 500 or 1000 nM) combined with 500  $\mu$ M SNP was evaluated. It was found that 50, 100, and 500 nM NAA resulted in an increasing trend in LR density in a dose-dependent manner, with the response to 500 nM NAA being significantly different compared with the control (Figure 3.11 (i)). However, 1000 nM NAA reduced LR density. No statistically significant difference was observed in the primordia density among the control and the NAA treatments, except the concentration of 1000 nM NAA where a significant increase was noted. However, there was no significant difference observed in the lateral plus primordia density among all the concentrations of NAA tested. The combinations of 500  $\mu$ M SNP with 50 or 100 nM NAA reduced the LR density compared with the respective NAA concentrations alone. The treatments of SNP with 500 or 1000 nM NAA completely inhibited LR formation.

In contrast, the treatments of NAA with SNP showed a dose-dependent increment in the primordia density. In addition, all the treatments resulted in a statistically significant difference in primordia density compared with the respective NAA concentrations alone (3.11 (ii)).

No statistical difference was observed in LR plus primordia density compared with various NAA concentrations alone or the control. Similarly, no statistical difference was observed in LR plus primordia density within the treatments of NAA with SNP. However, the treatments of NAA with SNP resulted in statistically significant difference compared with the respective NAA concentrations alone and the control (Figure 3.11 (iii)).

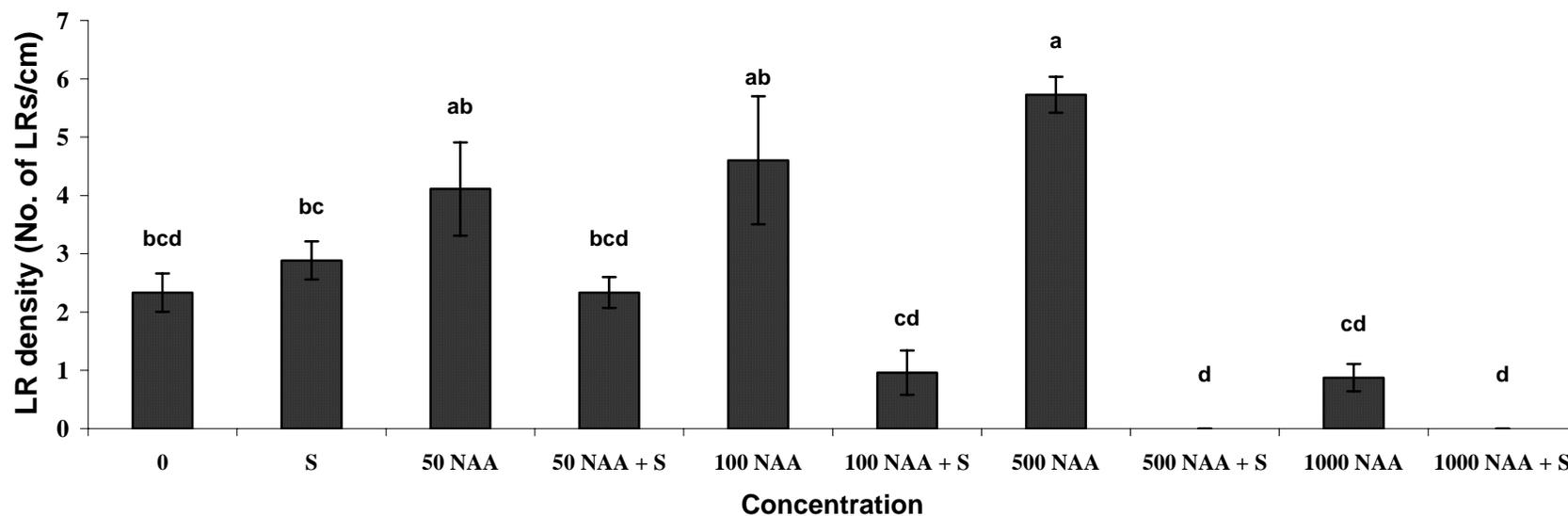
Among the different NAA treatments, only 1000 nM NAA significantly reduced the number of LRs compared with the control (Figure 3.12 (i)). The opposite response was observed in the number of primordia which was promoted in response to 1000 nM NAA (Figure 3.12 (ii)). No statistical difference was observed in the number of LR plus primordia density in all NAA concentrations tested (Figure 3.12 (iii)). A mixture of 500  $\mu$ M SNP with 50, 100, 500 or 1000

nM NAA decreased the number of LRs compared with the SNP alone and with the respective NAA concentrations. However, in response to SNP and NAA, the number of primordia dramatically increased compared with the control, SNP and the respective NAA concentrations alone. The treatments of NAA combined with SNP resulted in increased number of LRs plus primordia, but no statistical difference was observed among them (Figure 3.12).

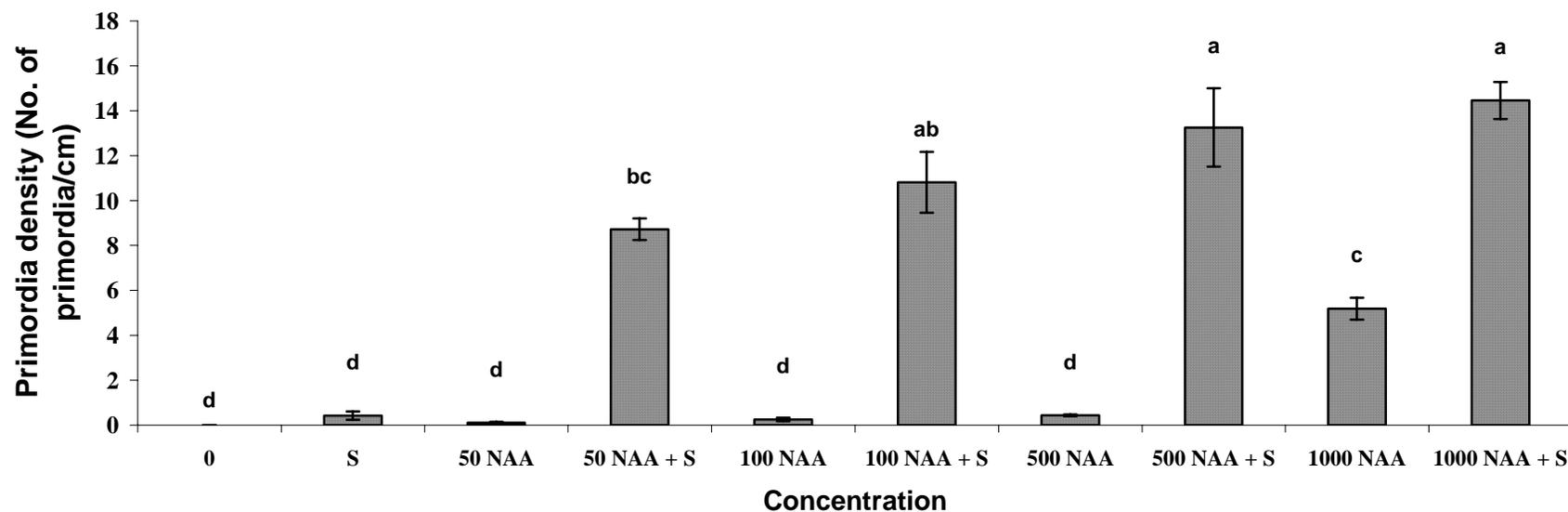
NAA decreased the PR length in a dose-dependent manner. The treatments of SNP with different concentrations of NAA resulted in no statistical difference compared with 500  $\mu$ M SNP. However, the treatments with SNP and 50 or 100 nM NAA reduced the PR length compared with the respective NAA concentrations alone. However, there was no statistical difference observed in the treatments with SNP and 500 or 1000 nM NAA (Figure 3.13).

The length of the longest LR was reduced at the concentration of 500 nM and 1000 nM NAA concentrations. The treatments of SNP with the different concentrations of NAA reduced the length of the longest LR compared with the respective NAA concentrations alone (Figure 3.14).

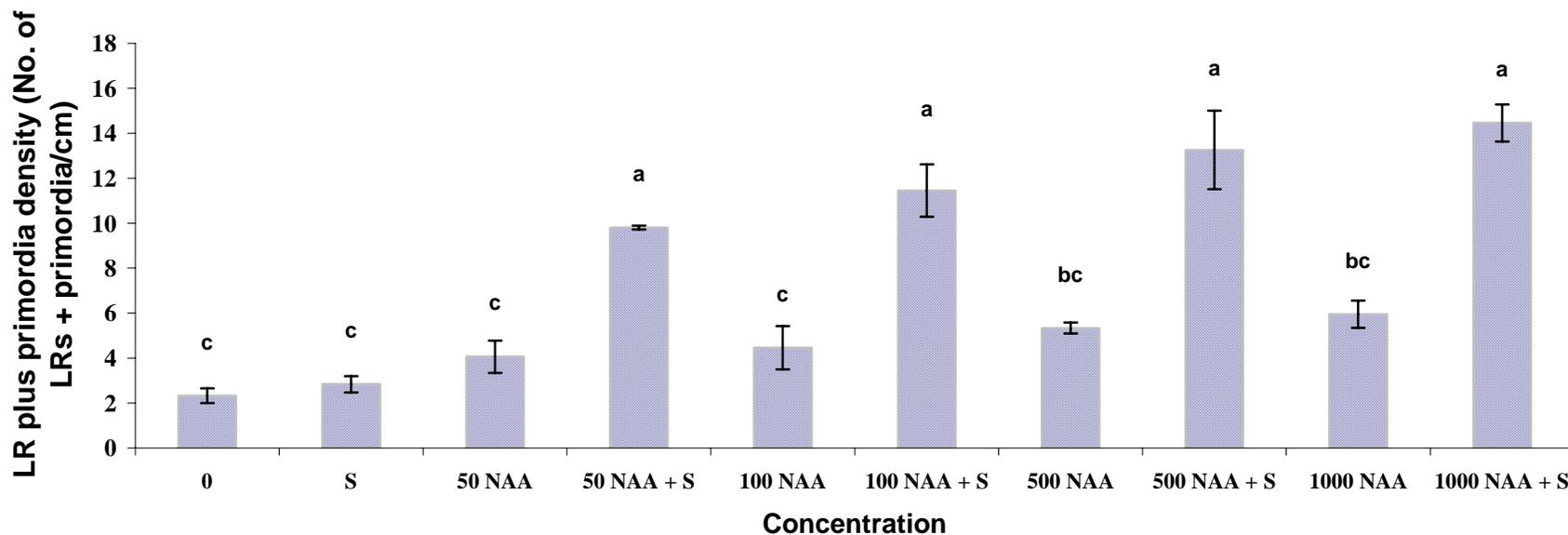
NAA alone decreased the apical distances of LRs and apical distances of LRs plus primordia in a dose-dependent manner compared with the control. Although they were not significantly different, a decreasing trend was observed in apical distances of LRs and apical distances of LRs plus primordia in the treatments of different concentrations of NAA with SNP, compared with the respective NAA alone (Figure 3.15). However, there was no difference observed in apical distance of LRs and apical distance of LRs plus primordia in the treatments of NAA and SNP, compared with the SNP alone. No statistical difference was observed in the apical distance of primordia in either NAA alone or in combination with SNP.



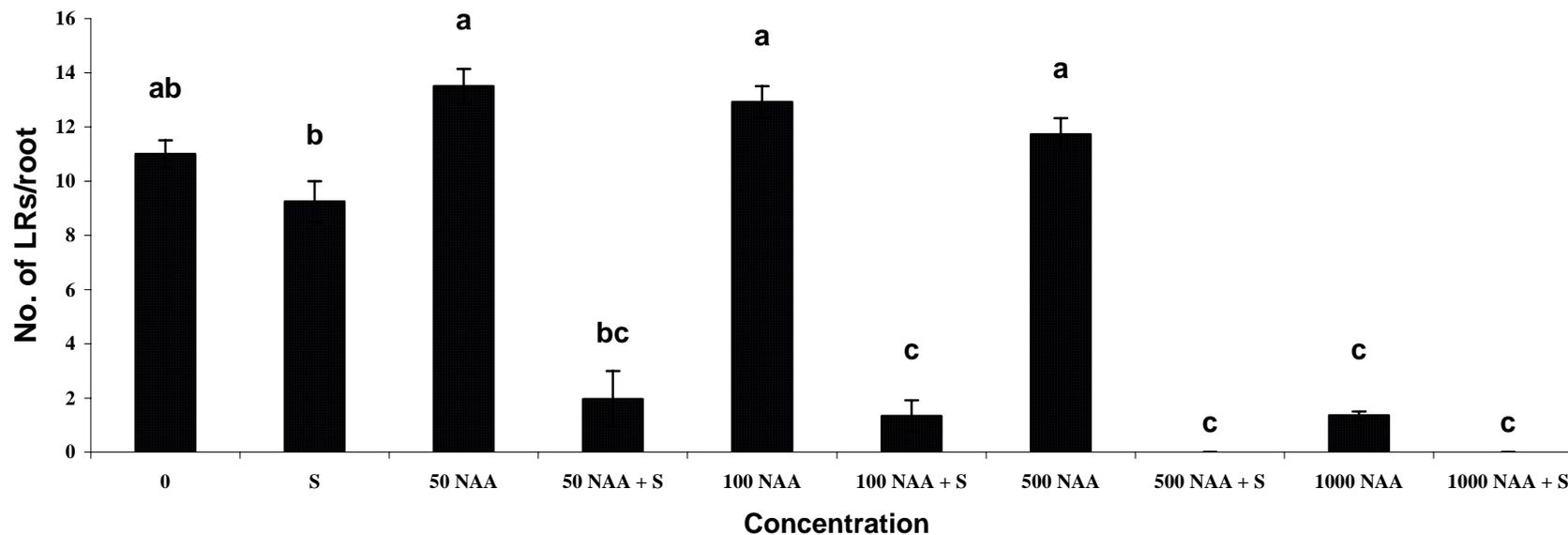
**Figure 3.11 (i):** Interaction between NAA and SNP on LR density. The effect of 500  $\mu$ M SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25 \pm 1^\circ\text{C}$ . S represents 500  $\mu$ M SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).



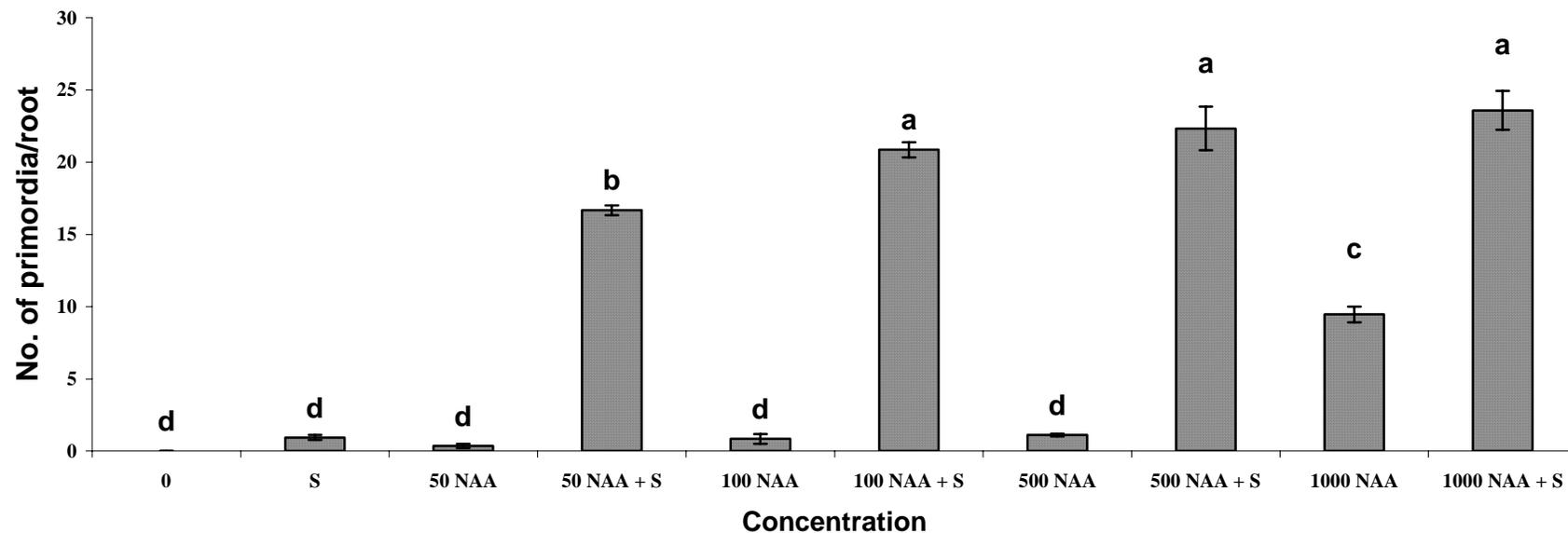
**Figure 3.11 (ii):** Interaction between NAA and SNP on primordia density. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



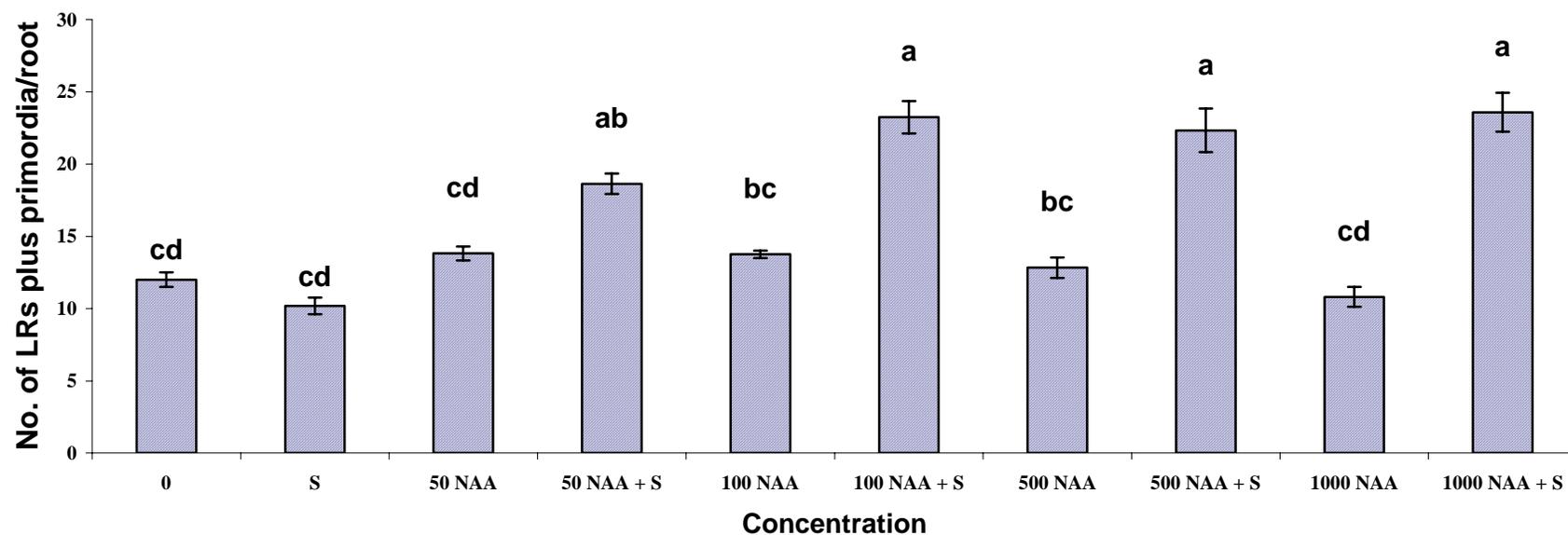
**Figure 3.11 (iii):** Interaction between NAA and SNP on LR plus primordia density. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25 \pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).



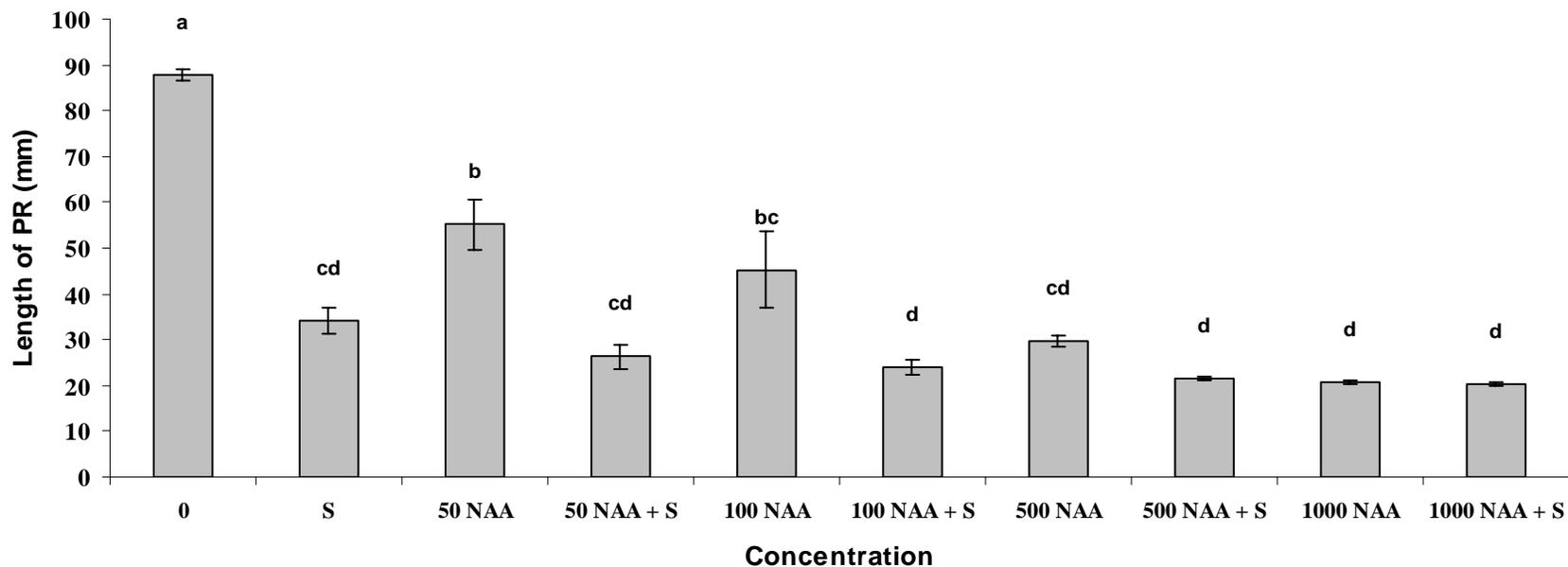
**Figure 3.12 (i):** Interaction between NAA and SNP on the number of LRs. The effect of 500  $\mu$ M SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu$ M SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



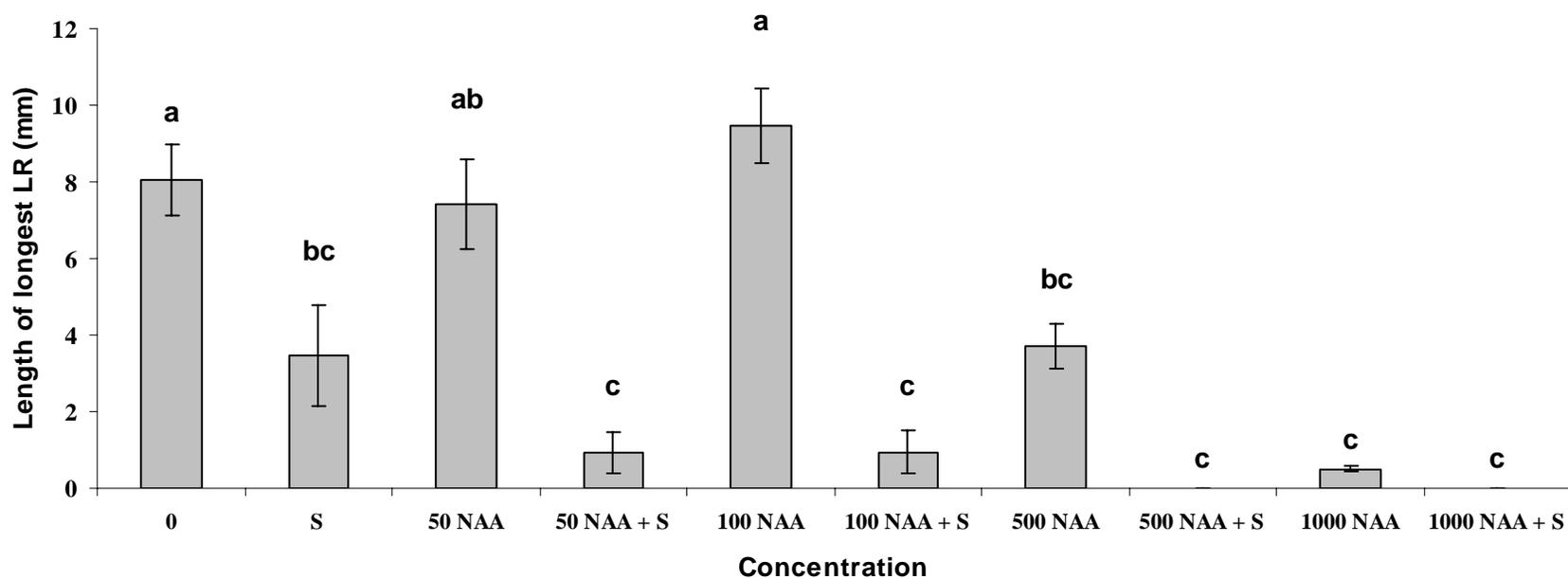
**Figure 3.12 (ii):** Interaction between NAA and SNP on the number of primordia. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



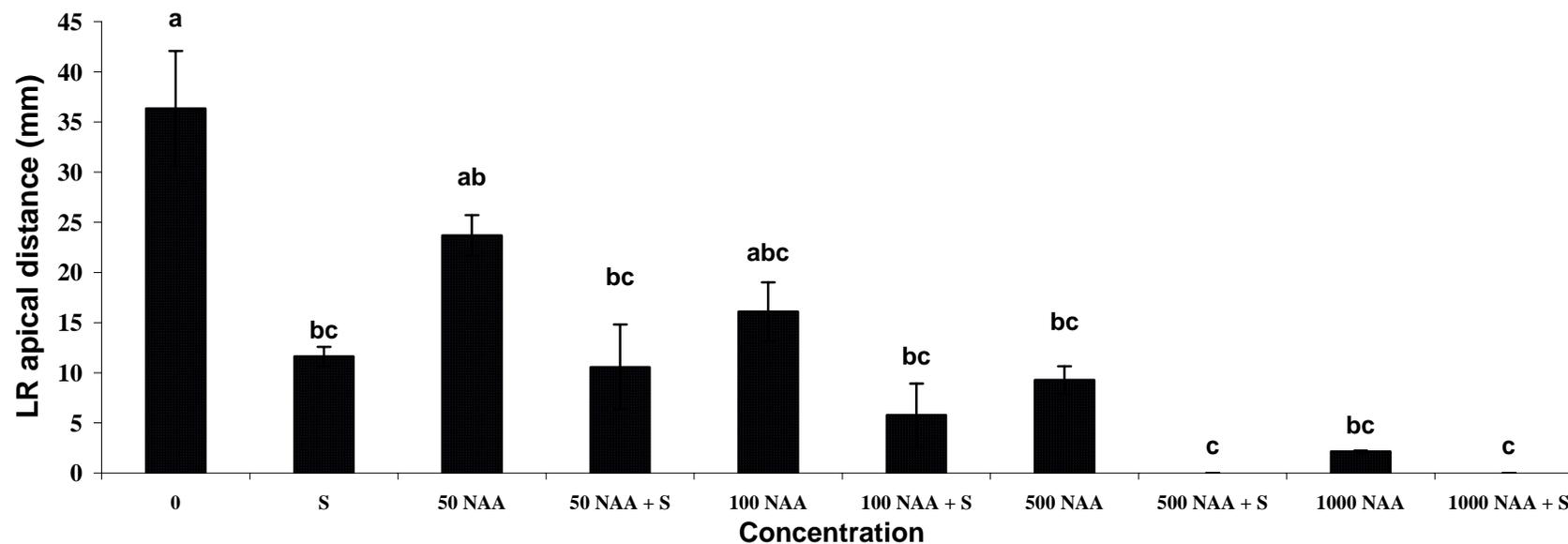
**Figure 3.12 (iii):** Interaction between NAA and SNP on the number of LR plus primordia. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



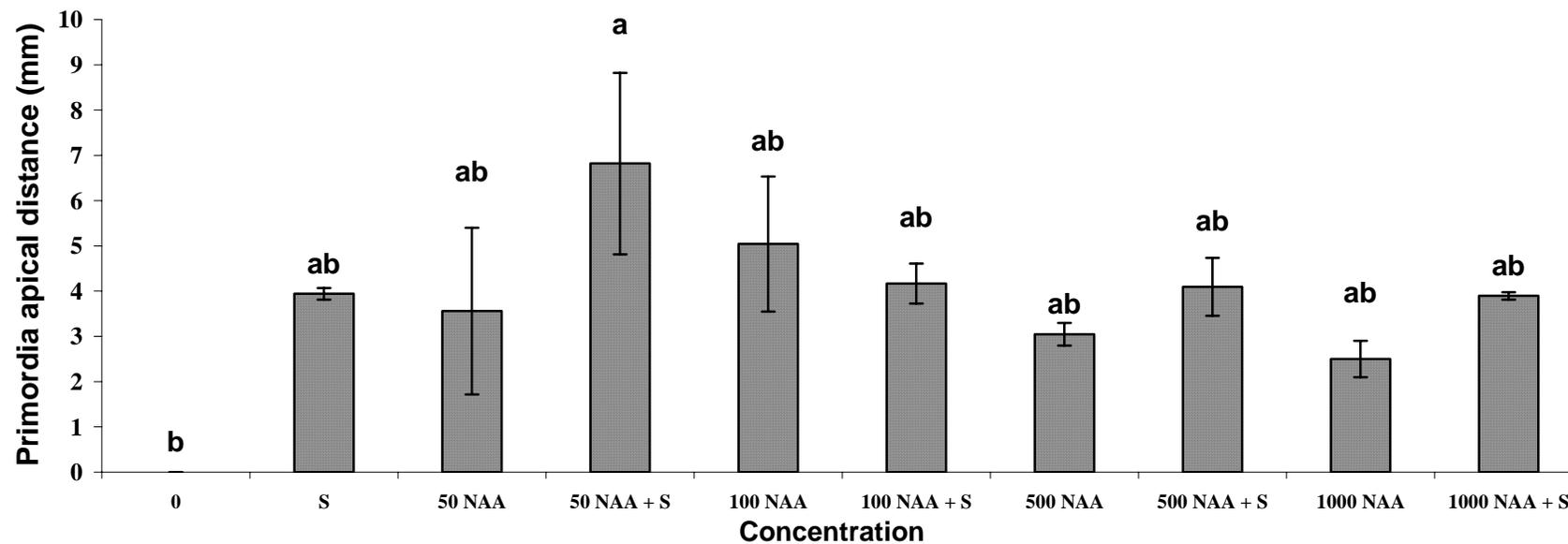
**Figure 3.13:** Interaction between NAA and SNP on the PR length. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p < 0.05$  (t-test).



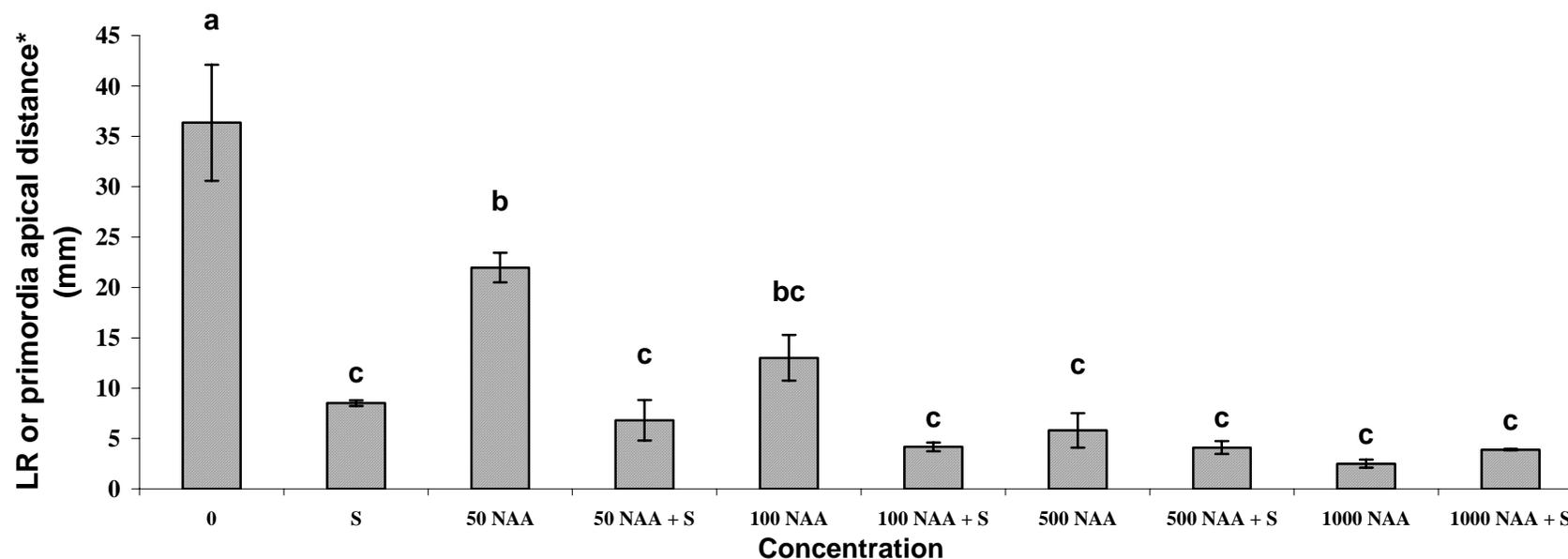
**Figure 3.14:** Interaction between NAA and SNP on the length of the longest LR. The effect of 500  $\mu$ M SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . S represents 500  $\mu$ M SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p < 0.05$  (t-test).



**Figure 3.15 (i):** Interaction between NAA and SNP on LR apical distance. The effect of 500  $\mu\text{M}$  SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25 \pm 1^\circ\text{C}$ . S represents 500  $\mu\text{M}$  SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).



**Figure 3.15 (ii):** Interaction between NAA and SNP on primordia apical distance. The effect of 500  $\mu$ M SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25 \pm 1^\circ\text{C}$ . S represents 500  $\mu$ M SNP. The values are means  $\pm$  SE from three independent experiments (n=16). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

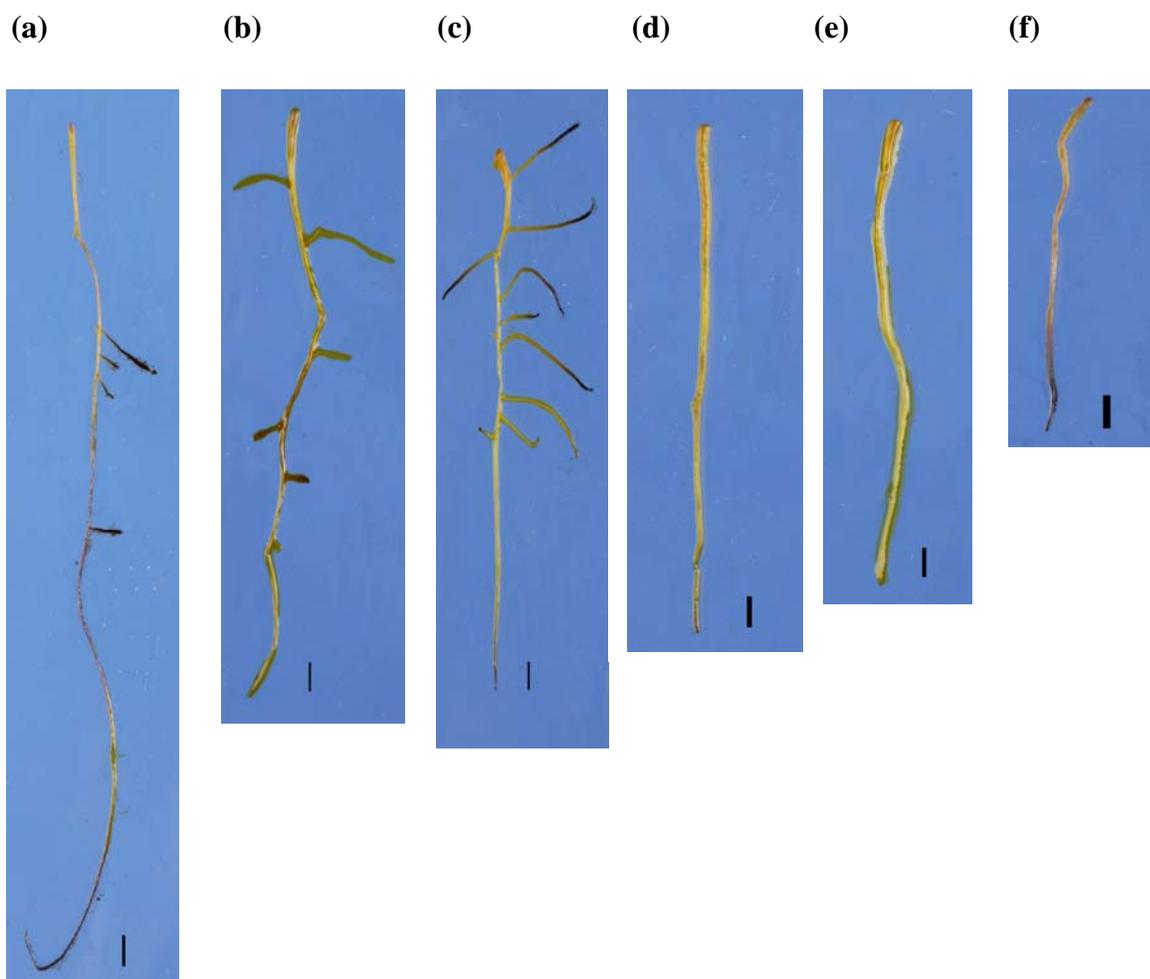


**Figure 3.15 (iii):** Interaction between NAA and SNP on LR or primordia apical distance. The effect of 500  $\mu$ M SNP and different concentrations of NAA (nM) on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25 \pm 1^\circ\text{C}$ . S represents 500  $\mu$ M SNP. The values are means  $\pm$  SE from three independent experiments ( $n=16$ ). Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

\* Distance to either first LR or primordia from the root rip.

### 3.9 The effect of CPTIO on SNP and NAA on excised root tips of tomato

Photographs of excised root tips treated with control, 500  $\mu$ M SNP and 50 nM NAA alone and together with CPTIO are shown in plate 3.6. Root tips appear brown in colour due to the staining process.



**Plate 3.5** Photographs of 2 cm long excised root tips of tomato treated with (a) 0  $\mu$ M SNP or NAA (Control), (b) 500  $\mu$ M SNP, (c) 50 nM NAA, (d) 1 mM CPTIO, (e) CPTIO+500  $\mu$ M SNP, (f) CPTIO+50 nM NAA and incubated for 7 days in complete darkness at  $25\pm 1^\circ\text{C}$ . Then roots were stained with 5% (W/V) chromium trioxide. Bar = 2 mm

The effect of CPTIO (1 mM) on SNP (500  $\mu$ M ) and NAA (50 nM ) were tested in 5 ml of culture media. Both SNP and NAA significantly increased the LR density, primordia density, LR plus primordia density compared with the control, but the promotion by NAA was greater than that of SNP (Figure 3.16 i, ii, iii). However, the NO scavenger, CPTIO, abolished the development of LRs or primordia on its own and in the presence of either SNP or NAA. No statistical difference was observed between the control and the CPTIO treatments. The effect on the number of LRs was also the same as on the LR density (Figure 3.17 i, ii, iii).

A statistically non-significant reduction in the PR length was observed in SNP, NAA compared with the control, but the CPTIO treatments alone or with either NAA or SNP significantly reduced the PR length compared with the control (Figure 3.18).

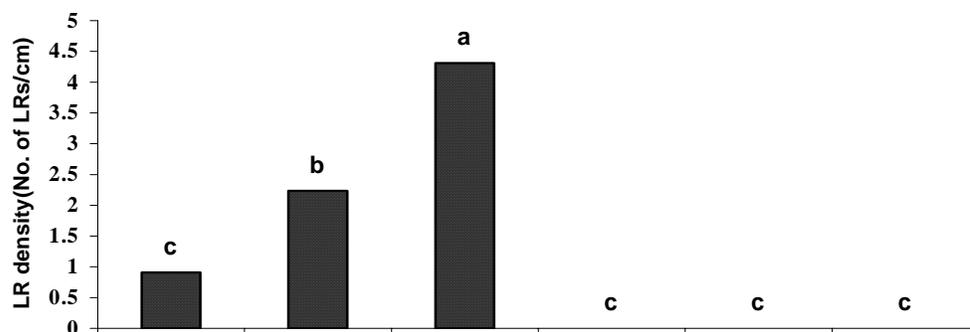
NAA and SNP increased the length of the longest LR compared with the control (Figure 3.19). NAA and SNP decreased both apical distances of LRs and apical distances of LRs plus primordia compared with the control. There were no apical distances for the treatments of CPTIO due to the absence of LRs (Figure 3.20 i, ii, iii).

**Figure 3.16:** The effect of CPTIO on SNP and NAA on the

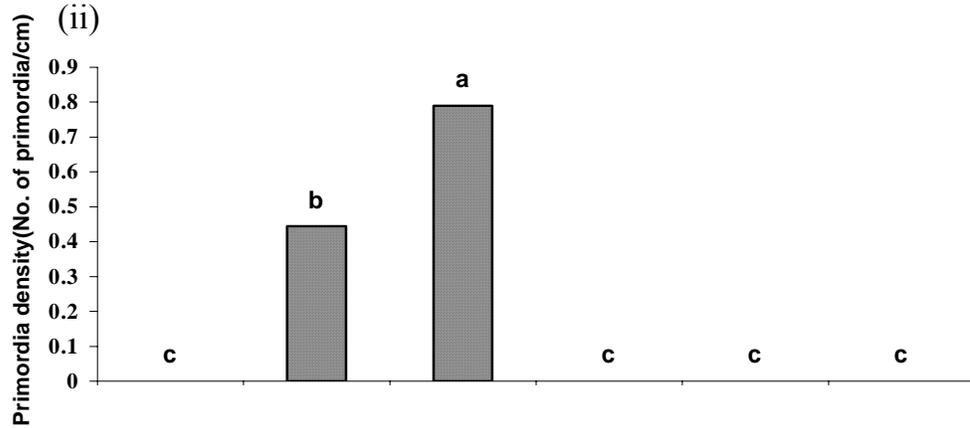
- (i) LR density
- (ii) Primordia density
- (iii) LR plus primordia density

The effect of 1 mM CPTIO on 500  $\mu$ M SNP and 50 nM NAA on 4-day-old, 2 cm long tomato excised root tips cultured in darkness at  $25\pm 1^\circ\text{C}$ . The values are means  $\pm$  SE from five replicates. Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).

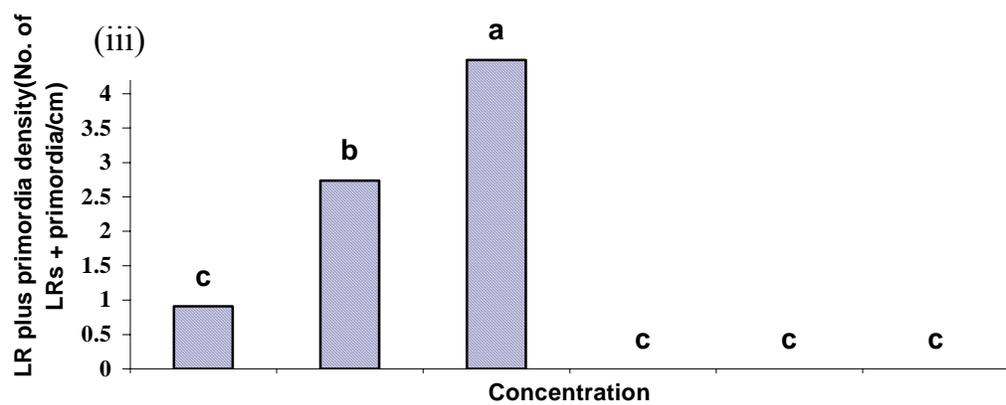
(i)



(ii)



(iii)

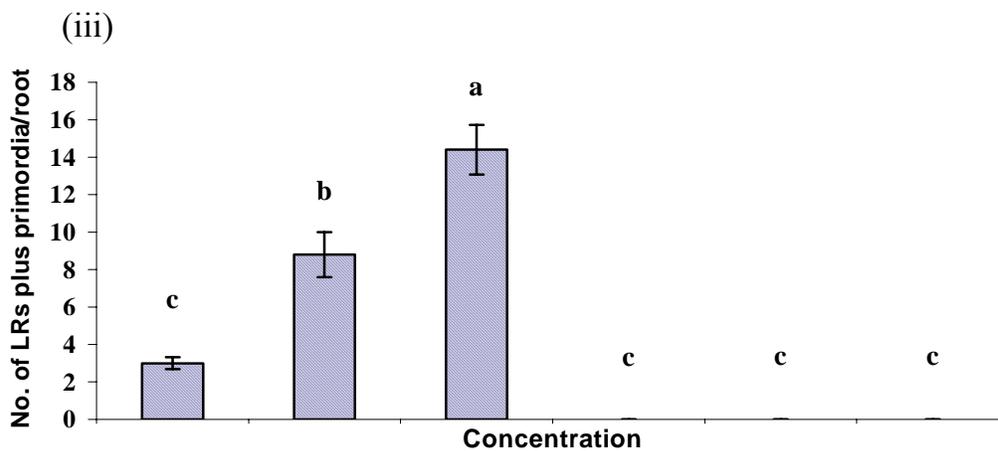
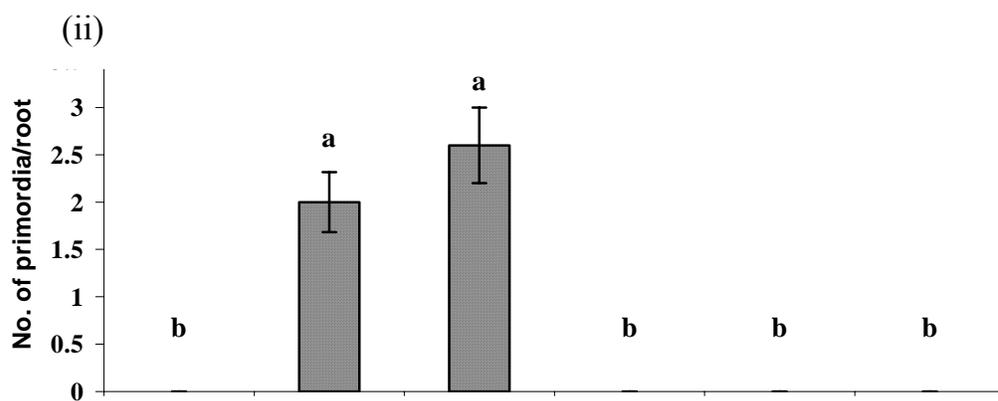
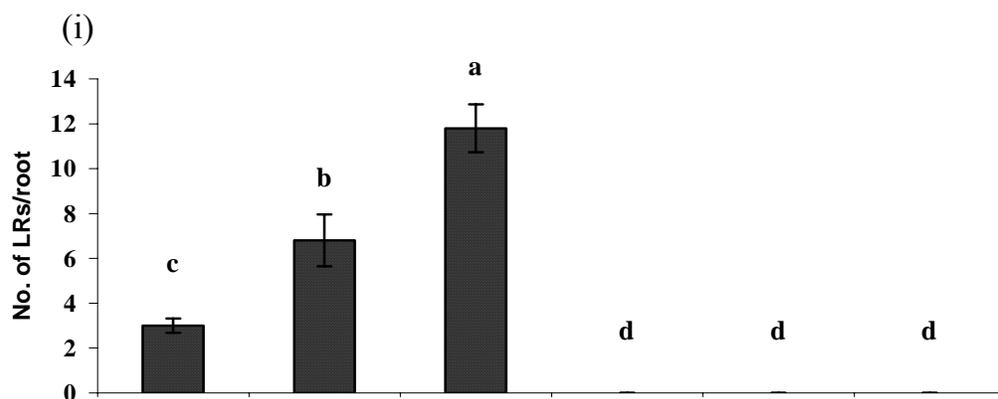


	1	2	3	4	5	6
500 $\mu$ M SNP	-	+	-	-	+	-
50 nM NAA	-	-	+	-	-	+
1 mM CPTIO	-	-	-	+	+	+

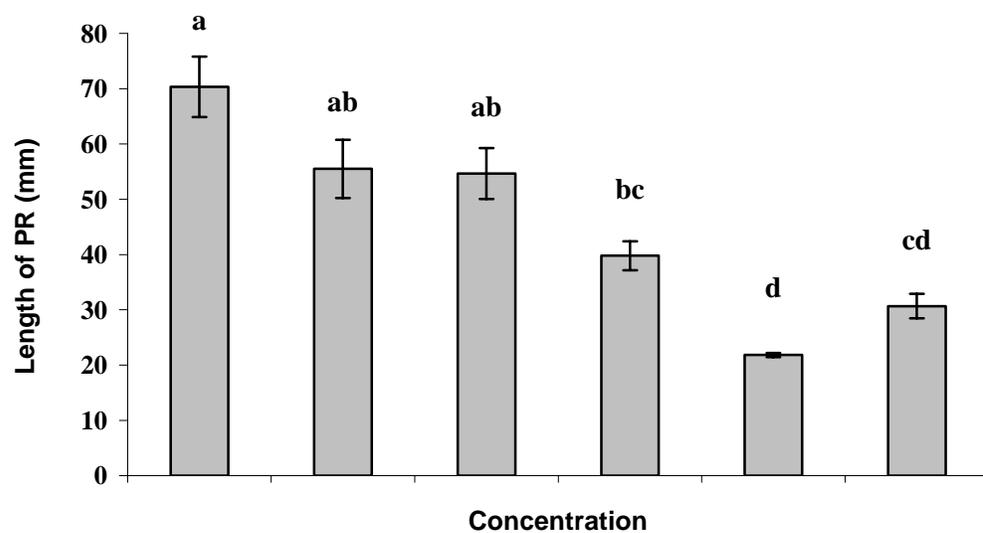
**Figure 3.17:** The effect of CPTIO on SNP and NAA on the.

- (i) Number of LRs
- (ii) Number of primordia
- (iii) Number of LRs plus primordia

The effect of 1 mM CPTIO on 500  $\mu$ M SNP and 50 nM NAA on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . The values are means  $\pm$  SE from five replicates. Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).

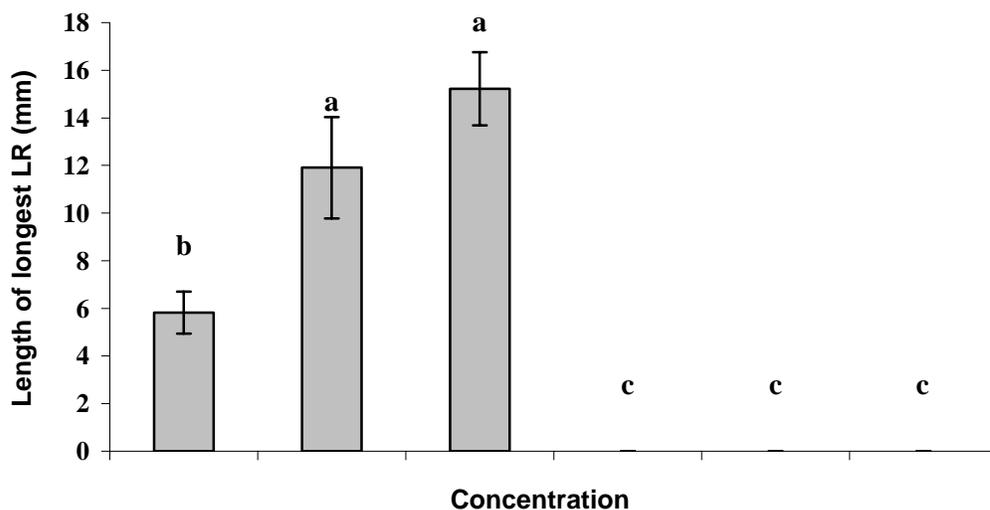


500 $\mu$ M SNP	-	+	-	-	+	-
50 nM NAA	-	-	+	-	-	+
1 mM CPTIO	-	-	-	+	+	+



500 $\mu$ M SNP	-	+	-	-	+	-
50 nM NAA	-	-	+	-	-	+
1 mM CPTIO	-	-	-	+	+	+

**Figure 3.18:** The effect of CPTIO on SNP and NAA on the PR length. The effect of 1 mM CPTIO on 500  $\mu$ M SNP and 50 nM NAA on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . The values are means  $\pm$  SE from five replicates. Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



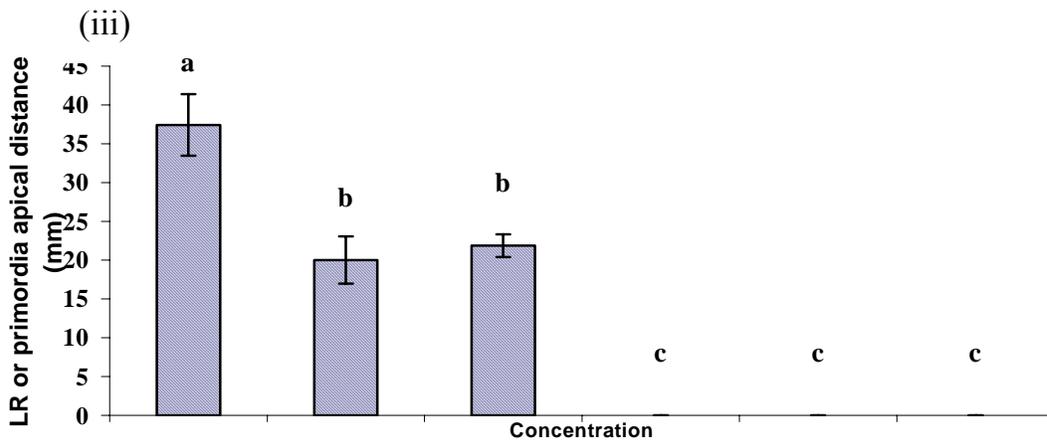
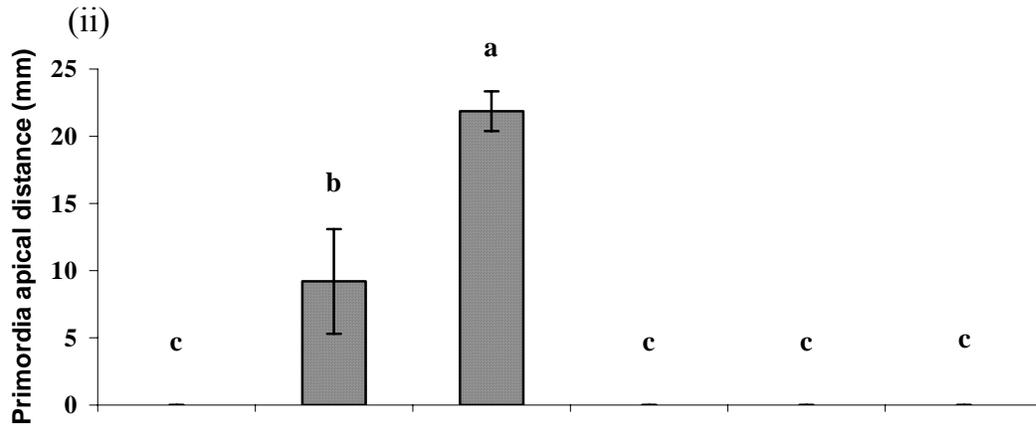
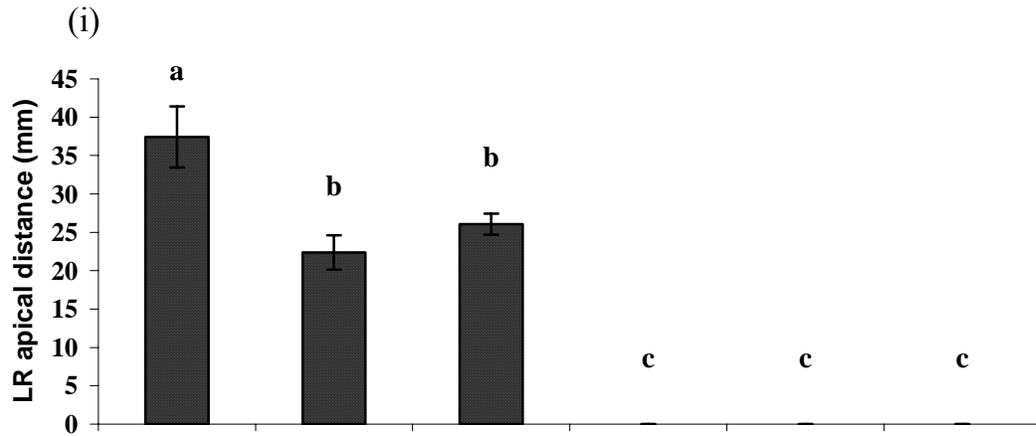
500 μM SNP	-	+	-	-	+	-
50 nM NAA	-	-	+	-	-	+
1 mM CPTIO	-	-	-	+	+	+

**Figure 3.19:** The effect of CPTIO on SNP and NAA on the length of the longest LR. The effect of 1 mM CPTIO on 500 μM SNP and 50 nM NAA on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at 25±1°C. The values are means ± SE from five replicates. Different letters indicate the significant difference with respect to each other at  $p \leq 0.05$  (t-test).

**Figure 3.20:** The effect of CPTIO on SNP and NAA on the

- (i) LR apical distance
- (ii) Primordia apical distance
- (iii) LR or primordia apical distance (distance to either first LR or primordia from the root rip).

The effect of 1 mM CPTIO on 500  $\mu$ M SNP and 50 nM NAA on 4-day-old, 2 cm long excised root tips of tomato cultured in darkness at  $25\pm 1^\circ\text{C}$ . The values are means  $\pm$  SE from five replicates. Different letters indicate the significant difference with respect to each other at  $p\leq 0.05$  (t-test).



500 $\mu$ M SNP	-	+	-	-	+	-
50 nM NAA	-	-	+	-	-	+
1 mM CPTIO	-	-	-	+	+	+

## **CHAPTER 4**

### **DISCUSSION**

For ideal exploitation of the beneath ground environment, it is important for a plant to have an optimally branched root system. Branching of roots occurs by endogenous initiation of new primordia which then eventually grow out of the main root to become secondary or LRs (Malamy and Benfey, 1997; Casimiro et al., 2003). It has been observed that a certain level of regularity in the spacing of LRs occurs along the parent root (Michael et al., 2006). Interactions between different signalling systems appear to be involved in the modulation of root architecture. Among the different signals are the plant hormones and more recently NO has been implicated as a key factor in LR initiation (Correa-Aragunde et al., 2006).

The results obtained in this study suggest that NO interacts with both NAA and ABA in the process of LR initiation and elongation. Most of the previous studies considered the effect of hormones on the number of LRs, LR density and the PR length (Hinchee and Rost, 1986; Correa-Aragunde et al., 2004; Creus et al., 2005). Studies on LR initiation and elongation are rather scarce compared with those on shoot elongation (Inada and Shimmen, 2000). Here we report the effect of NAA, ABA, the ABA biosynthesis inhibitor fluridone and the NO donor SNP alone as well as the combination of ABA or NAA with SNP. The interaction between CPTIO, a NO scavenger, and NAA or SNP is also reported.

#### **4.1 Preliminary experimental studies**

##### **4.1.1 Seedling roots versus excised root tips**

There was a reduction in LR density and the number of LRs in the control treatments (media only) of 1 cm long excised root tips compared with 1 cm long seedling roots cultured under a 14

h photoperiod. This could be explained with the findings of Laskowski et al., (1995), who showed that in 1 cm long root tips excised from 6 cm long radish seedling roots, an early LRP initiation (less than 3 cell layer stage) required auxin for its elongation into LR, but in the later stage of LRP initiation (3-5 cell layer stage), formation of a functional meristem was an auxin-autonomous event. At this stage, the LRP are either auxin-independent or they produce the required amount of auxin themselves. In our experiment, LR growth observed in the seedlings may be due to the auxin supply from the aerial part which was not available to the excised root tips, so that no LR elongation was observed in those treatments. However, Bhalerao et al., (2002) showed that the removal of apical segments at an early stage of seedling development (3-10 days after germination) had very little effect on LR initiation, but it inhibited LR emergence. Detailed microscopic analyses in the future would provide further detail on LRP initiation in the 1 cm long root tips used here and would determine whether the low number of LRs was due to a failure of LR elongation or not.

The influence of the aerial part of the seedling was further confirmed with the control treatments when 2 cm long seedling roots were cultured in complete darkness at  $25\pm 1^\circ\text{C}$  (Figures 3.1 a and b). These seedlings showed increased LR density and number of LRs compared to the excised root tips. This effect was previously suggested by Bhalerao et al., (2002) who showed that shoot-derived auxin is necessary for the LR elongation. Another study on both seedlings and excised roots of *Arabidopsis* showed that auxin synthesis rates and levels in excised roots were very much less than those in the seedlings (Ljung et al., 2005). Since the aerial part of the seedling influences root growth, excised root tips were used to study the involvement of NO, ABA and NAA on LR initiation and elongation in this thesis.

#### **4.1.2 The effect of initial length of the root segments**

LR elongation observed in the control treatment of 1 cm long excised roots tips was less than the same length of seedling roots suggesting that the root tips could be too young to be cultured. In a previous study, Barlow and Adam (1987) used 1 cm long excised root tips from tomato

seedlings, which were about 5-6 cm long. However, in this study, 1 cm long root tips were excised from the seedlings that were about 1.5 cm long after three days of germination.

Preliminary experiments also showed that 2 cm long excised root tips developed more LRs than the 1 cm long root tips. Most of the previous studies on excised root tips were carried out with 2 cm long apical segments. For example, 2 cm long root tips were excised from 4-day-old tomato seedlings (Hooker and Thorpe, 1998) and 2 cm long root apices were used to establish root cultures from *in vitro* grown apple root stock 'Jork 9', almond, plum and peach root stocks and their hybrids (Marin and Marin, 1998). Therefore it was decided to carry out the present study with 2 cm long excised root tips.

#### **4.1.3 The effect of light on LR elongation**

Light appears to affect the development of LRs, with the 1 cm long seedling roots and 1 cm long excised root tips responding differently under the 14 h photoperiod (Figure 3.2 and Table 3.1). Few of the excised root tips developed LRs and the LRs formed were small in number. More LRs developed in the 2 cm long excised root tips cultured under a 14 h photoperiod (data not shown) compared with the 1 cm long excised root tips, but this was still fewer than the 2 cm long excised root tips cultured in complete darkness. Limited lateral root development may, therefore, not be due to the initial length of the excised root tips as suggested in Section 4.1.2, but due to the inhibitory effect of light on LR elongation on excised root tips of tomato. In most plants, light appears to have an inhibitory effect on root elongation although in some it has a promoting effect (Seko and Nishimura, 1996). Therefore it was decided to carry out the experiments with 2 cm long excised root tips cultured in complete darkness at  $25\pm 1^\circ\text{C}$ .

#### **4.2 Confirmation of published data**

Before testing the interaction between ABA and NO, some of the important experiments in both of the previous studies of Hooker and Thorpe (1998) and Correa-Aragunde et al., (2004) were

reproduced in order to confirm the effect of ABA and NO, and in addition to extend the results to the excised root system of tomato. For further confirmation of the effect of ABA, fluridone, an ABA biosynthesis inhibitor, was also tested.

#### **4.2.1 ABA inhibits LR emergence**

The reduced LR density and the number of LRs on 2 cm long excised root tips of tomato treated with 1  $\mu$ M ABA (Figures 3.1 a and b) confirms the findings of Hooker and Thorpe (1998). In another study, ABA (10  $\mu$ M) exerted an inhibitory effect on the number of adventitious roots in the hypocotyl of rice plants (Steffens et al., 2006). However, in experiments with *Medicago truncatula*, a legume, 1  $\mu$ M ABA was found to promote LR density and the number of LRs (Liang and Harris, 2005). It was suggested that ABA exerts an opposite effect on LR emergence in legumes and non-legumes.

The length of the longest LR was reduced at 1  $\mu$ M ABA (Figure 3.1 d) suggesting that ABA regulates LR elongation on excised root tips. However, this effect of ABA was not observed in seedlings. This may be due to the influence of the aerial part of the seedlings contributing hormones, for example, cytokinin, which had a stimulatory effect on LR elongation in rice plants ([Debi et al., 2005](#)).

In the present study, the highest concentration of ABA (1  $\mu$ M) reduced PR length in tomato seedlings which is in agreement with the findings of Hooker and Thorpe (1998) in excised tomato root. In *Arabidopsis* seedlings, which were grown in the media of basic minerals, concentration of ABA up to 10  $\mu$ M had no effect on PR length (Smet et al., 2003), suggesting that different plants may respond differently in the regulation of PR elongation by ABA.

Experiments with fluridone, an ABA biosynthesis inhibitor, also support the earlier work of Hooker and Thorpe (1998). No difference was observed in LR density at the concentration of 1  $\mu$ M fluridone. However, an increase in primordia density and the LR plus primordia density,

leads to the suggestion that the effect of fluridone was mainly on LR initiation (Figure 3.2). An increase in the number of primordia supports this suggestion. This effect of fluridone may indicate that endogenous ABA was at sufficient concentration in the excised root to inhibit primordia initiation.

#### **4.2.2 SNP promotes LR density**

Based on the study on tomato seedlings (Correa-Aragunde et al., 2004), a range of concentrations of SNP were tested. Irrespective of whether seedling roots (Figure 3.2 a) or excised root tips (Figures 3.3 a, 3.4, 3.11) were used, SNP tended to increase LR density through an inhibition effect on the PR elongation (Figures 3.3 c, 3.6, 3.13) while SNP did not increase the number of LRs (Figures 3.2 b, 3.3 b and 3.5). In contrast, Correa-Aragunde et al. (2004) reported an increased number of LRs, within the range of 100-300  $\mu\text{M}$  SNP compared with the control. Possible explanations for the different observations include the response of different varieties of tomato ('Money maker' in the present study instead of 'Ace 55') to SNP and/or culture media used instead of distilled water. Higher concentration of SNP (2000  $\mu\text{M}$ ) reduced LR density (Figure 3.4). The inhibitory effect of SNP at higher concentrations had been reported previously (Garcia-Mata and Lamattina, 2002; Correa-Aragunde et al., 2004).

The effect of 500  $\mu\text{M}$  SNP on primordia initiation is shown in Figure 3.11(iii), suggesting that SNP promotes LRP confirming findings of other researchers (Celenza et al., 1995; Correa-Aragunde et al., 2006).

#### **4.2.3 NAA promotes LR initiation and elongation**

In a preliminary experiment, carried out with 2 cm long excised root tips, 100 nM NAA significantly promoted LR density and the number of LRs in excised root tips of tomato (Figures 3.9 a and b). The same result was obtained by Correa-Aragunde et al. (2004) using tomato

seedlings grown in dH<sub>2</sub>O and by Blakely et al. (1988) using excised root tips of radish grown in MS media. Although NAA promoted LR density the effect was reduced at higher concentration (1000 nM) of NAA (Figure 3.11(i)). Studies done by Correa-Aragunde et al. (2004) also reported a reduction in the number of LRs at higher concentrations of NAA in tomato seedlings.

However, the decrease in LR density at 1000 nM was matched by an increase in primordia density at 1000 nM suggesting that NAA was inhibiting LR emergence rather than LR initiation. This was further confirmed with LR plus primordia density not showing a significant difference across the NAA concentrations tested (Figure 3.11(iii)). The finding here is in agreement with those in other studies on tomato (Correa-Aragunde et al., 2006).

While NAA (100 nM) promotes LR density, the same concentration of NAA reduces the PR length in excised root tips of tomato. This is also found in a previous study (Correa-Aragunde et al., 2004). The reduction in apical distance at 100 nM NAA could be a result of more LR initiation along the length of PR and/ or a reduction in the PR length.

### **4.3 Interaction between NAA and NO**

The experiment testing 500  $\mu$ M SNP with different concentrations of NAA provided evidence of an interaction between NAA and NO on different parameters. The treatments of SNP with different concentrations of NAA resulted in a dose-dependent reduction in LR density compared with the respective NAA concentrations suggesting a possible inhibitory effect in response to the combined action of the two plant growth regulators. This is similar to the inhibitory effect of a high concentration of SNP (2000  $\mu$ M) or NAA (1000 nM) observed in Figures 3.4 and 3.11. For example, the effect of 1000 nM NAA on LR elongation (either in LR density or the number of LRs) was the same as the addition of 100 nM NAA with 500  $\mu$ M SNP.

Another possibility for the above effect is that the NO (released from SNP) could be inhibiting either LR initiation or LR elongation. Taking the primordia density into consideration, it would

seem that there was mainly inhibition of LR elongation since a dose-dependent increase was observed in the primordia density in the treatments of SNP with different concentrations of NAA compared with the respective NAA concentration alone. No statistical difference was observed in the LR plus primordia density among all the treatments of NAA with SNP. However, as the number of LR plus primordia was always significantly greater in the SNP with NAA treatments than the control, SNP or NAA treatments alone, this suggests that NO may be a limiting factor in LRP initiation.

Semi-quantitative RT-PCR analysis done by Correa-Aragunde et al., (2006) showed that NAA and SNP application target some of the same genes: NAA induced the expression of four different cell cycle regulatory genes whereas SNP mainly induced two of those genes and one to a lesser extent.

#### **4.4 NO operates downstream of ABA**

ABA has been shown to inhibit LR development (Signora et al., 2001). However, the intermediate reaction mechanism is not known. NO is a free radical involved in many different biological pathways and previously it was shown that NO could interact with ABA in stomatal guard cells (Neill et al., 2002; Garcia-Mata and Lamattina, 2002). Here, we report for the first time that NO released via SNP interacts with ABA in the regulation of LR elongation.

In order to test whether ABA and SNP interact with each other in the process of LR development, an experiment was designed to assess the relationship between the two different factors. The inhibitory effect of ABA on LR density was counteracted by the external application of SNP (up to 1400  $\mu$ M). SNP had a similar but lesser effect in counteracting the reduction in the number of LR's caused by application of ABA.

No statistical difference was observed in the PR length, the length of the longest root or apical distance between the treatments of ABA with different concentrations of SNP and the respective

SNP concentrations alone suggesting that these decreases were due to the SNP alone. This supports a previous finding that 1  $\mu$ M ABA has no significant effect on the PR length.

Overall, ABA did not relieve the inhibitory effects of SNP, but SNP could relieve the inhibitory effects of ABA on LR density and LR number, suggesting that NO, released from SNP, was acting downstream of ABA

#### **4.5 The effect of CPTIO on SNP and NAA**

The effect of SNP was prevented by the addition of CPTIO, the NO scavenger, suggesting that the LR initiation or elongation was due to the NO, which was released from SNP. The experiment with CPTIO confirms other studies showing that NO is necessary for the initiation or elongation of LR (Pagnussat et al., 2002; Correa-Aragunde et al., 2004). In addition, CPTIO prevented LR initiation and elongation of the NAA-treated roots suggesting that NO plays a role in the NAA mediated LR initiation and elongation. The arrest by CPTIO on SNP or NAA-treated excised roots could be in either LR initiation or LR elongation. No primordium initiation was observed in the microscopic analysis suggesting that the effect of CPTIO was on the initiation of LR. Therefore, it seems that the effect of NO and NAA is mainly on LR initiation. This key finding was recently corroborated by Correa-Aragunde et al. (2006), whose paper was published after this research was begun.

Since it has been shown that NO and NAA are involved only in the LR initiation, some other factor must be involved in the regulation of LR elongation. This factor could be cytokinin which showed a stimulatory effect on LR elongation on rice plants (Debi et al., 2005). This hypothesis is related to the finding of Debi et al., (2005) showing that LR elongation promoted by cytokinin was reduced with the addition of exogenous auxin, suggesting that the promoting effect of cytokinin on LR elongation could be inhibited by the exogenous auxin such as NAA. Similarly

this argument can be applied to the effect of the treatments of SNP and the concentrations of NAA.

Another possibility for reduced LR density in the treatments of SNP with the concentrations of NAA is that the initiated LRP may need a minimum space in between them in order to develop as LR. The increased number of LRP developed within the reduced PR length makes it more compact and difficult to elongate as LR. This idea coincides with recent research which showed a positional mechanism in regulating the initiation of LRP in *Pinus taeda*. In this study, it was found that the presence of a LRP guarantees the initiation of the other primordium in a different pole (Michael et al., 2006).

In order to reduce the cost, the CPTIO experiment was carried out with a single root tip in 5 ml of media in a glass vial instead of two root tips in 25 ml media in a Petri dish. The modification of the experimental system might have had an effect on the results. Preliminary experiments were carried out to compare the treatments of SNP, NAA and the control in the Petri dishes versus the 5 ml media in glass vials. Although the root tips in the Petri dishes grew better than those in the vials, the expected significant differences among the different treatments were observed in both of the two experimental systems. Therefore it was decided to carry on with the CPTIO experiments in the vials.

Experiment with CPTIO alone abolished the initiation of LR, probably due to the removal of internal NO by CPTIO. All the treatments with CPTIO failed to develop LRP suggesting that the concentration of CPTIO used (1mM) might be too high and possibly toxic. However, 1 mM CPTIO was used in a previous study (Correa-Aragunde et al., 2004) to test the effect of 200  $\mu$ M SNP on tomato seedlings and while those treatments developed limited LRs there were fewer than the control.

## 4.6 Conclusions

The root system in a higher plant is an essential part of the plant. The elongation of LR is a highly coordinated process that incorporates both intrinsic pathways that determine features of a species and response pathways that coordinate environmental stimuli, which modulate the intrinsic pathways. The more we understand the interactions between these pathways the more information we have to modulate the root architecture.

Since the preliminary experiments showed that the aerial part of the seedling influenced root growth, excised root tips were used to study the involvement of NO, ABA and NAA on LR initiation and elongation in this thesis. In addition, light appeared to affect the development of LRs on the cultured root tips. Therefore it was decided to carry out the experiments with 2 cm long excised root tips cultured in complete darkness at  $25\pm 1^\circ\text{C}$ .

Irrespective of whether seedling roots or excised root tips were used, NO (released from SNP) tended to increase LR density through an inhibition effect on the PR elongation while SNP did not increase the number of LRs. However, SNP promoted LRP confirming findings of other researchers (Celenza et al., 1995; Correa-Aragunde et al., 2006).

NAA showed a dose-dependent increase in LR density which eventually reduced at higher concentrations. However, NAA showed similar potency to initiate LRs regardless of NAA concentration gradient, that is, no difference in LRs plus primordia density across the NAA concentrations tested.

The present research provided a better understanding of the interactions between NAA and NO on the LR initiation and elongation in excised root tips of tomato. Although, NAA showed a dose-dependent increase in LR density, the treatments of SNP with different concentrations of NAA resulted in a dose-dependent reduction in LR density compared with the respective NAA concentrations suggesting a possible inhibitory effect in response to the combined action of the

two plant growth regulators. However, there was mainly inhibition of LR elongation since a dose-dependent increase was observed in the primordia density in the treatments of SNP with different concentrations of NAA compared with the respective NAA concentration alone. No statistical difference was observed in the LR plus primordia density among all the treatments of NAA with SNP.

The concentration of 1  $\mu$ M ABA reduced LR density and the number of LRs on excised root tips of tomato. Experiments with fluridone, an ABA biosynthesis inhibitor, may indicate that endogenous ABA was at sufficient concentration in the excised root to inhibit primordia initiation. The present research also provides new information, for the first time, on the interaction between NO and ABA on the LR density on the excised root tips of tomato. ABA did not relieve the inhibitory effects of SNP, but SNP could relieve the inhibitory effects of ABA on LR density and LR number, suggesting that NO, released from SNP, was acting downstream of ABA.

The inhibition of LR initiation and elongation observed with the treatment of CPTIO and SNP or NAA confirmed that NO plays a key role in NAA-mediated LR elongation.

Our results strongly support the effect of NO as a versatile molecule, providing evidence for the involvement of NO in the initiation of LRs rather than the elongation. This key finding was recently corroborated by Correa-Aragunde et al. (2006), whose paper was published after this research was begun.

#### **4.7 Directions for future study**

Present research showed that ABA interacts with NO in the regulation of LR elongation. Within the limited time, this study considered the effect of ABA and NO on LR density, number of LRs developed, PR length, apical distance and LR length. However, information from this study is limited as this did not include the effect on primordia density. Therefore, future study should be

done considering both LR and primordia density, which would give a better understanding of the interaction between ABA and NO.

Light seems to promote LR elongation in some plants while it inhibits on others. For example light promotes root elongation in rhizome fragments of *Helianthus tuberosus* (Gautheret, 1969). Here, in this research, light inhibited LR elongation on cultured root tips of tomato. The inhibitory effect of light could be in either LR initiation or LR elongation. Testing the effect of light on primordia initiation would give a better understanding of light on excised roots.

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## Appendix 1

### White's medium stock solution:

#### White's major salt (10×)

<u>Chemical</u>	<u>g/l</u>
Ca(NO <sub>3</sub> ) <sub>2</sub>	2.0428
KNO <sub>3</sub>	0.81
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.74
KCl	0.65
KH <sub>2</sub> PO <sub>4</sub>	0.12

Dissolve these chemicals in 750ml of dH<sub>2</sub>O and bring the volume up to 1 L.  
Label and store at 4<sup>0</sup>C.

#### Organic supplement (100×)

<u>Chemical</u>	<u>g/500ml</u>
Myo-inositol	5
Nicotinic acid	0.025
Pyridoxine – HCl	0.025
Thiamine HCl	0.005
Glycine	0.1

Dissolve in 400ml of dH<sub>2</sub>O and bring the volume up to 500ml.  
Label and store at 4<sup>0</sup>C.

#### Iron stock (100×)

Soln A – FeSO <sub>4</sub> .7H <sub>2</sub> O	1.39g in 200ml dH <sub>2</sub> O
Soln B – Na <sub>2</sub> EDTA.2H <sub>2</sub> O	1.865g in 200 ml dH <sub>2</sub> O
Mix soln A and soln B	

Adjust to 500 ml.

Store the stock soln in dark bottle at 4<sup>0</sup>C.

**Preparation of White's Media:**

	<u>200ml</u>	<u>500ml</u>	<u>1L</u>	<u>2L</u>
dH <sub>2</sub> O - ml	150	375	750	1500
Major salt (10×) - ml	20	50	100	200
Organic supplement (100×) - ml	2	5	10	20
Iron stock (100×) - ml	2	5	10	20
1. Mix.				
2. Add sugar	1.6g	4g	8g	16g
3. Adjust pH 5.6±0.1				
4. Adjust volume to (with dH <sub>2</sub> O)	200ml	500ml	1L	2L
5. Dispense				
6. Autoclave				

## Appendix 2

**(1) Work out the weight of ABA you need to have in the medium (a given volume):**

For 500 ml medium containing  $10^{-6}$ M ABA,

M.W of ABA = 264.3

Amount of ABA in 1M solution	=	264.3 g in 1 liter
$10^{-6}$ M ABA	=	$264.3 \times 10^{-6}$ g in 1 liter
for 500 ml media containing $10^{-6}$ M ABA	=	$264.3 \times 10^{-6} \times 500/1000$ g
the weight of ABA to be added	=	$264.3 \times 5 \times 10^{-1}$ $\mu$ g

**(2) Prepare the stock solution:**

13.21mg	in	50 ml dH <sub>2</sub> O
therefore $26.42 \times 10^{-2}$ mg	in	1 ml
Concentration of stock	=	$26.42 \times 10^{-2}$ $\mu$ g in 1 $\mu$ l

**(3) Work out the volume of the stock solution to be added to the medium that will give the required weight of ABA in a given volume of the medium:**

		$264.3 \times 5 \times 10^{-1}$ $\mu$ g
The volume of stock to be added =	=	—————
		$26.42 \times 10^{-2}$ $\mu$ g/ $\mu$ l
		= 500 $\mu$ l

Similarly, the weight of ABA for 500 ml media containing  $10^{-7}$ M ABA can be calculated.

Weight of ABA to be added	=	$264.3 \times 10^{-7} \times 500/1000$ g
		$= 264.3 \times 5 \times 10^{-2}$ $\mu$ g

		$264.3 \times 5 \times 10^{-2}$ $\mu$ g
The volume of stock to be added	=	—————
		$26.42 \times 10^{-2}$ $\mu$ g/ $\mu$ l
		= 50 $\mu$ l

### Appendix 3

**(1) Work out the weight of SNP you need to have in the medium (a given volume):**

For 500 ml medium containing SNP,

M.W of SNP = 297.95

Amount of SNP in 1M solution	= 297.95g in 1 liter
One $\mu\text{M}$ ( $10^{-6}\text{M}$ ) SNP	= $297.95 \times 10^{-6}$ g in 1 liter
for 500 ml media containing $10^{-6}\text{M}$ SNP	= $297.95 \times 10^{-6} \times 500/1000$ g
the weight of SNP to be added	= $297.95 \times 0.5 \times 10^{-6}$ g

**(2) Prepare the stock solution:**

0.5958g	in	10 ml dH <sub>2</sub> O
therefore 0.05958g	in	1 ml

**(3) Work out the volume of the stock solution to be added to the medium that will give the required weight of SNP in a given volume of the medium:**

$$\text{Required amount of SNP for } 1 \mu\text{M solution} = \frac{297.95 \times 0.5 \times 10^{-6} \text{ g}}{0.05958 \text{ g/ml}}$$

$$= 0.25 \times 10^{-2} \text{ ml}$$

$$= 2.5 \mu\text{l}$$

For;

50 $\mu\text{M}$ SNP	—————>	$2.5 \times 50$	= 125 $\mu\text{l}$
100 $\mu\text{M}$ SNP	—————>	$2.5 \times 100$	= 250 $\mu\text{l}$
300 $\mu\text{M}$ SNP	—————>	$2.5 \times 300$	= 750 $\mu\text{l}$
500 $\mu\text{M}$ SNP	—————>	$2.5 \times 500$	= 1250 $\mu\text{l}$

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