CHAPTER 12

Chemical Treatments and Its Impact on Seedling Emergence and Vigour

12.1 Introduction

In-situ conservation within protected habitats must remain the primary means to conserve plant species. Plants are very sensitive to the environmental changes around them. The habitat disturbance and change in habitat conditions, ultimately leading to habitat loss, due to undesirable growth of unwanted plant species over important plant species, devoid them of required ecological conditions. This would result in fragmentation of the population thereby restricting its distribution to smaller pockets and finally wiping the species from the entire area. At this crucial point, *ex-situ* mechanism plays an important role in conserving endangered plant species (Badola and Pal, 2002) through their recovery, habitat rehabilitation and restoration. *Ex situ* conservation helps to provide the flexibility in responding to unforeseen environmental changes. The risk of extinction of threatened species in nature can be reduced by reinforcing the *ex-situ* raised individuals (Bowes, 1999). Expensive techniques may not be essential for successful seed germination and developing healthy individuals for some species, but identifying suitable technique, based on species requirements, would minimize the wasting of the resources (Fay, 1994; Benson *et al.*, 2000; Butola and Badola, 2004b, 2007). For raising mass planting material in species conservation programme, especially for Himalayan herbs, the best option would be the production of uniform and vigorous seedlings using identified pre-sowing chemical treatments (Butola and Badola, 2004b; 2006a, 2007). Also, high and rapid germination determine good stand establishment which results in higher yields. Therefore, fast and uniform germination is equally important for superior crop production (Afzal *et al.*, 2002).

The genus *Swertia* (Gentianaceae) is represented by over 170 species globally, out of which over 40 species occur in Indian Himalayan region and 13-14 species are recorded in Sikkim Himalaya (India). Amongst them, *Swertia chirayita* is of great

importance for its several biologically important compounds (Singh, 2008) and its multiple utilities in local medicine (Pradhan and Badola, 2008). Species bears high pharmaceutical value in national and international markets (Badola and Pal, 2002). Plant possesses anti-inflammatory, hypoglycemic, anti-fungal, anti-bacterial, anti-malarial, anti-oxidant (Joshi and Dhawan, 2005) and anti-pyretic (Bhargava *et al.*, 2009) properties; and can be used as natural larvicide (Balaraju *et al.*, 2009). *S. chirayita* is considered to be a bitter tonic, febrifuge and laxative and is used in fever, burning of body, intestinal worms and skin diseases etc., in a variety of forms and in combination with other medicines. In ancient Indian literature, *Madhavachikitsa* text, chapter *Jvarachikitsa*, one of the most important post *Caraka Sushruta Samhita* (*Caraka Sushruta* era: between 5^{th}-10^{th} century) mentions effectiveness of *S. chirayita* in *Jvara* or fever (Mishra, 2009). As entire plant possesses medicinal properties, the *S. chirayita* is uprooted unsustainably in wild at very early stage of development or before seeds are fully matured. Such human pressures have led to its endangerment in nature and has been assessed as critically endangered (Ved *et al.*, 2003b; IUCN, 2008). Amongst the prioritized species, *S. chirayita* further recommended at the top for its conservation through *ex-situ* cultivation by an international forum of experts (Badola and Pal, 2002). For both strengthening *in-situ* conservation and promoting *ex-situ* cultivation, availability of standardized propagation techniques and quality planting material of targeted species is a pre-requisite (Butola and Badola, 2004b, 2006a, 2007). To maintain sustainability of genetic stock (seeds), it would be equally important to use seeds from *ex-situ* sources (Butola and Badola, 2007)

The present investigation was carried out with the aim to fulfill ever-increasing demand of the pharmaceuticals and to strengthen *in-situ* species recovery programme by developing and standardizing suitable technique to improve seedling emergence and raise vigorous and mass scale planting material for *Swertia chirayita*. The objective of the present study were, (i) to examine the effect of earlier laboratory tested and identified best pre-sowing chemical treatments on nursery seedling emergence, emergence rate, growth and biomass; (ii) to examine variability amongst seed sources on above parameters in nursery conditions, and (iii) to identify appropriate morphological traits in screening vigorous seedlings in *S. chirayita*.

In the present work, we chose two earlier tested best chemical treatments in improving seed germination potential in a seed germinator, using the same *ex-situ* sources for obtaining seeds, as a continuum (second phase) of earlier work (refer chapter 11) in nursery condition with addition of assessment on seedling growth and vigour in *S. chirayita*.

12.2 Materials and methods

12.2.1 Seed collection

Mature seeds of *Swertia chirayita* (Roxb. ex Fleming) H. Karst.were harvested during December from six experimental set ups viz., four in natural habitats (1. shrubberies, 2. forest-slope, 3. open-slope and 4. tree-canopy) and two in nursery conditions, on soil amended beds using garden soil and forest humus (1. open net-shade, and 2. temperature controlled green-house; $25^0C \pm 5^0C$) [refer chapter 11]. Seeds were mixed well individually for each source, room dried for 15 days and stored in air tight specimen tubes at 4^0C until the experimentation.

12.2.2 Experimental design

During May 2008, seeds from all the six sources were provided with the best chemical treatments, which we had earlier tested and identified in laboratory on germination in *S. chirayita* seeds collected from the same plots (refer chapter 11). In the previous experiment, all four concentrations of gibberellic acid resulted in high percent seed germination and GA_3 (250 µM) most effectively stimulated seed germination for majority of the sources (open slope, tree canopy, net shade, green house); and amongst three concentrations used for $NaHClO_3$, the 5minutes soaking time gave best results; while, all the three concentrations of KNO_3 were less effective in stimulating seed germination, except for shrubberies, due to which KNO_3 was not considered in the current experiment. The seed lots from each source were divided into three sets containing 150seeds/set/source. The first set of seeds was soaked in GA_3 (250µM) for 24hrs and the second set soaked in $NaHClO_3$ for 5minutes. The third set was soaked in double distilled water (DDW) for 24 hrs which served as control. The soaked seeds for each set were washed thoroughly with DDW and sown

(0.5cm depth in rows; maintaining 5cm distance between each seed in three replicates; 50seeds each per replicate) in nursery trays containing a mixture of garden soil, sand and crushed farm yard manure (pH: 7.02) in equal volume. The experiment was performed under the net-house (Temperature, max: 25.04^{0}C, min: 13.22^{0}C; Relative humidity, max: 91%, min: 65%) located in the Institute campus at an altitude of *ca* 2000m, in a completely randomized design. Prior to seed sowing, the soil mixture was wetted with water in order to avoid washing away of seeds from the trays. Taking in account the importance of soil moisture in seedling emergence, careful watering was done as per the necessity.

Daily observation on seedling emergence was made using a powerful hand lens for 60 days. To avoid competition, thinning was done after 16 weeks of seed sowing, maintaining maximum distances between each seedling (10-12 cm). After 24 weeks of seed sowing, 5 seedlings for each treatment per seed source from experimental trays were randomly harvested and assessed for growth parameters, including the total dry biomass. As the plant remains in the rosette form for consecutive two year, the plant height was not taken into consideration. The root length was determined from the root collar to the tip of the primary root. The collar diameter was measured using a digital calliper (Mitutoyo, Japan). The dry biomass was determined by oven-drying seedlings to constant weight at 70^{0}C.

12.2.3 Statistical analysis

Multivariate analysis of variance (MANOVA; $\alpha = 0.05$) in general linear model (GLM) was carried out to determine the effect of seed sources, treatments and their interaction on seedling emergence variables and morphological traits using SPSS 10.0 software (SPSS Inc. 1989). A Bonferroni test ($p<0.05$) was used to determine the differences amongst mean values for different treatments. Mean days for emergence (M_d) was calculated according to the equation of Edmond and Drapala (1958) as: $M_d = \sum nd/\sum N$, where, "n" is number of newly emerged seedlings in each day "d", and "N" is total number of seedlings emerged at the end of the test. Seedling emergence rate was calculated using modified Timson index of germination velocity by Khan and Ungar (1984) as: $T = \sum G/t$, where "G" is percentage of seeds emerged after 2

days interval and "t" is the total emergence period. To identify the most effective treatment for producing healthy seedlings, seedling vigour index (SVI) was calculated following Butola and Badola (2004b), SVI = (d/M_d)*100, where, "d" is dry weight/seedling; "M_d" is seedling mean days of emergence and 100 is constant value. In order to analyze the relationship between different morphological traits and to identify convenient morphological traits to select vigorous seedlings, Pearson's correlation (r) analyses were performed by pooling data from all the treatments and the relationship amongst different seedling traits was examined.

12.3 Results

12.3.1 Seedling emergence

Table 27 depicts the result of multivariate analysis of variance (MANOVA) showing effect of treatments, seed sources and their interaction on different seedling emergence variables. Different treatments showed significant effect on all the seedling emergence parameters ($p<0.0001$).

Seedling emergence in all the sources and treatments including control started within 18 to 22 days of seed sowing and maximum number of seedlings emerged within 56 days of sowing. Both GA_3 (250μM) and $NaHClO_3$ (5 minutes) highly stimulated the seedling emergence in all seed sources over control; however GA_3 was more effective than $NaHClO_3$ (Figure 38). Over 59% seedling emergence was recorded with GA_3 in all the seed sources and the maximum latitude in stimulation was recorded in seeds sourced from forest slope, where seedling emergence was promoted from 6% (control) to 69% (GA_3). Comparatively, 40% seedling emergence was the maximum value recorded in seeds (green house) treated with $NaHClO_3$.

Seedling emergence rate was faster in GA_3 followed by $NaHClO_3$ in all the seed sources (Table 28). With GA_3, amongst six sources, the seedling emergence was faster for green house (11.53±0.47) and slower for net-shade (9.51±2.39), comparatively. With $NaHClO_3$, the seedling emergence was faster for tree canopy (4.52 ± 1.35) and shrubberies (4.51 ±0.93) compared to other sources.

12.3.2 Seedling growth and biomass

MANOVA revealed significant ($p<0.0001$) effect of seed sources and treatments on all the seedling growth parameters; however, the effect of their interaction was non-significant for number of leaves and root diameter (Table 29). Amongst the seed sources, forest slope resulted in significantly ($p<0.05$) higher values for root length, total plant dry biomass and seedling vigour index compared to other sources (Figure 39, 40 and 41). Significantly ($p<0.05$) minimum seedling growth was achieved for shrubberies. Significantly ($p<0.05$) lesser number of leaves was recorded for the seed sourced from open slope compared to other sources. GA_3 and $NaHClO_3$ treatment significantly ($p<0.05$) enhanced all the seedling growth parameters over control; however the effect of $NaHClO_3$ was in-significant for number of leaves. On comparision, GA_3 appeared significantly ($p<0.05$) more effective over $NaHClO_3$ treatment for all the growth parameters including total plant dry biomass and seedling vigour index. All the plant growth parameters showed significant correlation with each other except number of leaves (Table 30).

12.4 Discussion

In a species, variation in germination behaviour between sites (Baskin and Baskin, 1998; Ceraboloni *et al.*, 2004) is applicable not only to the natural populations but also to the *ex-situ* produced seeds under different conditions (refer chapter 10). Present study, with *ex-situ* produced seeds in *S. chirayita,* indicates the germination variability amongst the seed sources in addition to existence of physiological dormancy (refer chapter 10).

Both GA_3 (Mehanna *et al.*, 1985; Tipirdamaz and Gomurgen, 2000; Butola and Badola, 2004ab, 2006a; Chandra *et al.*, 2006) and $NaHClO_3$ (Fieldhouse and Sasser 1975; Hsiao 1979; Drew and Brocklehurst 1984; Bewely and Black, 1994; Butola and Badola, 2004ab, 2006a, 2007) are known for their stimulatory action on seed germination, breaking seed dormancy and increasing germination rate. In the present study, both GA_3 (250μM) and $NaHClO_3$ (5minutes) treatments were stimulatory to seedling emergence and over all seedling growth for all six *ex-situ* seed sources. The

commencement of seedling emergence was significantly faster ($p<0.05$) in $NaHClO_3$ treatment followed by GA_3 over control. Such differences in seedling emergence between treated and untreated seeds might be due to alteration of physiological process by the liberated enzymes inside the embryo (Kattimani *et al.*, 1999). Although, the mean days of seedling emergence were higher in GA_3 than $NaHClO_3$, the faster seedling emergence rates for GA_3 indicated it as more effective treatment than $NaHClO_3$ (Table 28).

GA_3 proved to be more effective than $NaHClO_3$ in stimulating seedling emergence in *S. chirayita* in all six *ex-situ* seed sources in the present study, however, higher effectiveness of $NaHClO_3$ over GA_3 was observed in case of *Angelica glauca* and *Heracleun candicans* (Butola and Badola, 2004b, 2006a). In present study, greatly stimulated seedling emergence by using higher concentration of GA_3, i.e., 250µM is comparable to *Picrorhiza kurrooa,* where Chandra *et al.*, (2006) achieved marked improvement in seed germination (78.3% compared to 1.7% in control). The inhibitory effect of higher soaking period in $NaHClO_3$ (10 and 15 minutes) was observed in *S. chirayita* having smaller seed size (refer chapter 10), whereas, increased soaking period resulted in higher seed germination in the case of *A. glauca* and *H. candicans* with large seeds (Butola and Badola, 2004b, 2006a). This has indicated that the seed size might play a vital role in deciding concentration of chemicals or growth regulators to be used for achieving best results. Such variability in Himalayan herbs indicates species specific nature of seeds. As in *S. chirayita*, GA_3 stimulated seed germination and germination rate in many plant species is known (Nicolas *et al.*, 1996; Koyuncu, 2005; Cetinbas and Koyuncu, 2006). Similar stimulatory effect of $NaHClO_3$ in seed germination has been reported (Clevering, 1995; Yildiz and Celal, 2002; Butola and Badola, 2007).

Higher seedling emergence in *S. chirayita* with GA_3 in the present study might be due to amylase synthesis in seeds (Amen, 1968; Galtson and Davies, 1969). GA_3 stimulates hydrolytic enzymes that are needed for the degradation of the cells surrounding the radical and thus speeds up germination by promoting seedling elongation (Karssen *et al.*, 1989; Rood *et al.*, 1990; Silva *et al.*, 2004). GA_3 also

regulates hypocotyl growth altering the extent of hypocotyl cell elongation (Cowling *et al.*, 1999) and plants response to external environment (Chakrabarti and Mukherji, 2003). However, Chen and Chang (1972) argued against the contention that gibberellic acid stimulates seed germination via amylase synthesis, taking case of dormant wild oat *(Avena fatua)* seeds, which confirmed that amylase activity increases following post germination growth.

Scarification or bleaching of seed coat by $NaHClO_3$ (Vujanovic *et al.*, 2000; Bohm, 2003) or other modification in seed coat (Hsaio, 1979) may not be the cause of stimulation of seedling emergence in *S. chirayita*, as the delicate seeds are enclosed in very thin seed coat which do not need extra physical effort in promoting germination. It may be due to either increase permeability of seed coat to oxygen through the removal of phenolics (Hurley *et al.*, 1989) or release of oxygen with decomposition of $NaHClO_3$, thus enhancing oxidative respiration, thereby, promoting seed germination (Bewely and Black, 1994; Vujanovic *et al.*, 2000).

Other factor which might be responsible for stimulation of seedling emergence by $NaHClO_3$ are decomposition of germination inhibitors (Mackinnon and Alderton, 2000), overcoming dormancy by lowering of pH thereby promoting oxygen uptake (Bohm, 2003), or promoting an enhancement of α-amylase activity (Kanecko and Morohashi, 2003). Soaking seeds in $NaHClO_3$ for lesser time (5 minutes) appears relatively useful to achieve successful recruitment of *S. chirayita*. $NaHClO_3$ not only protects the seeds from pathogens in nursery conditions but also facilitates leaching of toxic compounds and improves the uptake of water and oxygen contributing to increase seed germination or seedling emergence.

In the present study, GA_3 application resulted to higher seedling dry biomass and seedling vigour index. This may be due to the fact that the action of GA_3 applied before sowing could have sustained itself till the plants reached vegetative stage. Further, this could be related to an elaboration of an endo-membrane system and regulated synthesis of proteins required for germination, which in turn could have contributed additionally to the amino acid rescue and protein turnover during active

metabolism, later in plant life (Shah, 2007). $NaHClO_3$ is applied as a suitable disinfectant for seed surface sterilization (Bewley and Black, 1994; Kaneko and Morohashi, 2003) and also known to promote seed germination and overcomes seed dormancy (Bewely and Black, 1994). However, $NaHClO_3$ effect on seedling growth (Fieldhouse and Sasser, 1975; Chun *et al.*, 1997; Butola and Badola, 2004b, 2006a, 2007) and its mechanism has rarely been reported.

All the seedling growth parameters were positively correlated with each other but the correlation was non-significant for some of the growth parameters. Both collar diameter and root diameter though showed significant correlation with all the growth parameters except number of leaves; collar diameter is considered strongest morphological trait for identifying seedling vigourousness in *S. chirayita* because root being present below ground surface is not conveniently visible. Further, the collar diameter is always larger than the root diameter (*personal observation*) which does not necessitates going further step to identify vigorous seedlings.

12.5 Conclusion

The present study suggests that stimulatory effect of GA_3 (250μM) (preferably) and $NaHClO_3$ (5 minutes) on seedling emergence and seedling vigour may solve the problem of increasing pressure on the wild population of species for raw material, as a tool to strengthen *ex-situ* cultivation of *S. chirayita*. Higher seedling emergence rate obtained with GA_3 (250μM) may lead to early establishment of the seedlings, which bear the ability to compete and withstand the rough climate. This could be advantageous during the process of species reinforcement in the nature using individuals raised by *ex-situ* means. While reinforcing the species into the natural habitats, collar diameter should be used for identifying the vigourous seedlings which would not only save time but also minimize the wastage of the resources (Fay, 1994; Benson *et al.*, 2000; Butola and Badola, 2004b). Such step will definitely reduce the risk of extinction of endangered species (Bowes, 1999) like *S. chirayita* in Himalaya.

Table 27. Results of MANOVA showing effect of different seed sources, treatments and their interaction on seedling emergence variables in *Swertia chirayita*.

Dependent variable	Independent variable					
	Source (S)		Treatment (T)		S x T	
	F	Sig.	F	Sig.	F	Sig.
Seedling onset time (days)	6.902	.000	9.581	.000	2.633	.016
50% emergence time (days)	6.784	.000	17.562	.000	3.887	.001
Final emergence time (days)	10.176	.000	456.074	.000	8.152	.000
Seedling emergence (%)	2.526	.046	689.359	.000	2.622	.017
Mean days of emergence (days)	9.231	.000	103.197	.000	9.558	.000
Emergence rate	0.954	.458	378.929	.000	1.026	.442

Table 28. Index of emergence rate in *Swertia chirayita*, using a modified Timson Index of germination velocity.

Source	Treatment		
	Control	GA_3 (250µM)	$NaHClO_3$ (5 minutes)
Shrubberies	1.27 ± 0.35^{a}	10.20 ± 0.75^{a}	4.51 ± 0.93^{a}
Forest slope	0.20 ± 0.17^{b}	10.34 ± 1.38^{a}	4.43 ± 0.47^{a}
Open slope	0.48 ± 0.13^{bc}	11.33 ± 1.49^{a}	3.27 ± 0.96^{a}
Tree canopy	0.69 ± 0.19^{ab}	11.08 ± 2.53^{a}	4.52 ± 1.35^{a}
Net shade	1.02 ± 0.13^{ac}	09.51 ± 2.39^{a}	2.99 ± 0.69^{a}
Green house	0.54 ± 0.23^{bc}	11.53 ± 0.47^{a}	4.28 ± 0.65^{a}

Values in each column with the same superscript are not significantly different at $p>0.05$, Bonferroni test ($\pm$: standard deviation).

Table 29. Results of MANOVA showing effect of seed sources, treatments and their interaction on different growth parameters in *Swertia chirayita.*

Dependent variable	Independent variable					
	Source (S)		Treatment (T)		S x T	
	F	Sig.	F	Sig.	F	Sig.
Collar diameter	40.429	.000	99.950	.000	3.839	.000
Leaf number	9.842	.000	11.358	.000	0.626	.787
Largest leaf length	78.377	.000	95.652	.000	7.250	.000
Largest leaf width	24.729	.000	26.920	.000	4.089	.000
Root diameter	13.780	.000	12.200	.000	0.662	.755
Root length	138.446	.000	182.174	.000	4.929	.000
Seedling dry biomass	311.877	.000	262.684	.000	43.899	.000
Seedling Vigour Index	328.761	.000	148.523	.000	33.030	.000

Table 30. Correlation matrix (Pearson Product-Moment Correlation Coefficient) between different growth parameters in *Swertia chirayita*.

	Number of leaves	Collar diameter (mm)	Largest leaf length (mm)	Largest leaf width (mm)	Root diameter (mm)	Root length (mm)	Seedling dry biomass (gm)	Seedling Vigour Index
Number of leaves	1.000							
Collar diameter (mm)	0.149^{ns}	1.000						
Largest leaf length (mm)	0.304**	0.717**	1.000					
Largest leaf width (mm)	0.113^{ns}	0.693**	0.887**	1.000				
Root diameter (mm)	0.202^{ns}	0.694**	0.709**	0.654**	1.000			
Root length (mm)	0.233*	0.677**	0.707**	0.607**	0.620**	1.000		
Seedling dry biomass (gm)	0.127^{ns}	0.591**	0.753**	0.730**	0.485**	0.697**	1.000	
Seedling Vigour Index	0.094^{ns}	0.558**	0.718**	0.705**	0.456**	0.678**	0.989**	1.000

** $p<0.01$, * $p<0.05$, ns: non significant

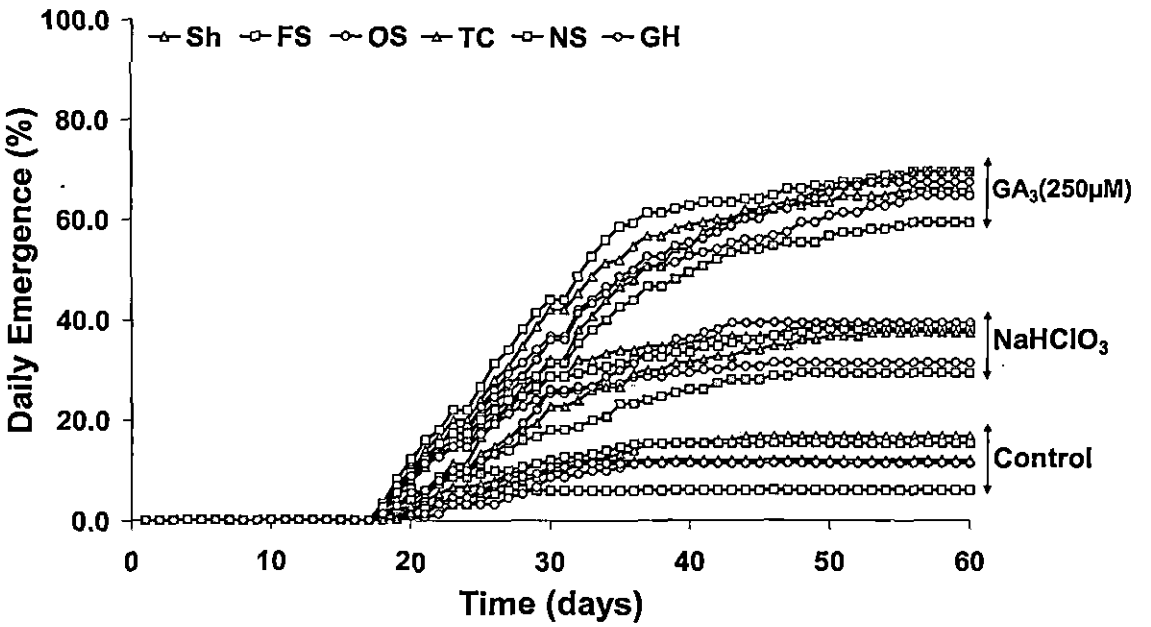

Figure 38. Effect of different seed sources and pre-sowing chemical treatments on seedling emergence in *Swertia chirayita*; Sh: shrubberies, FS: forest slope, OS: open slope; TC: tree canopy; NS: net shade; GH: green house

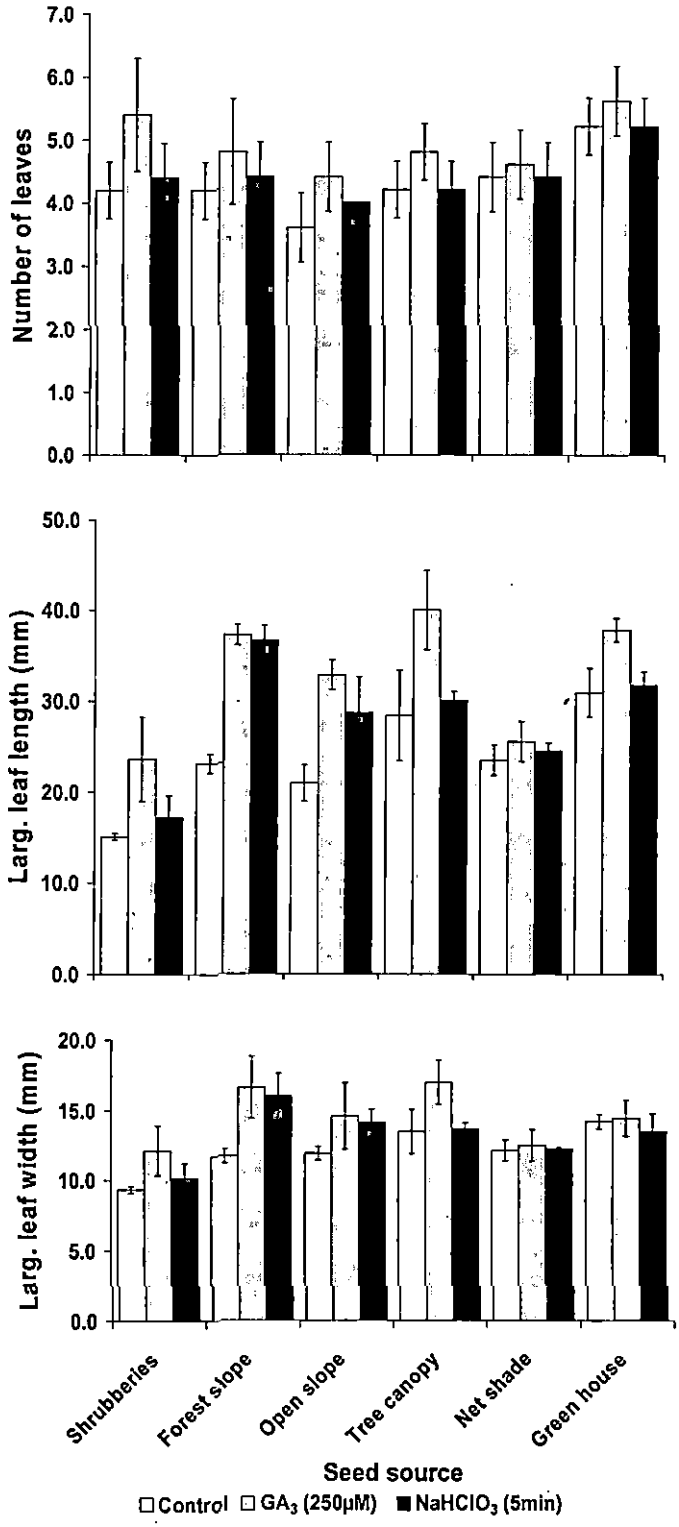

Figure 39. Effect of pre sowing chemical treatments on number of leaves, largest leaf length and width in *Swertia chirayita* (error bars: standard deviations)

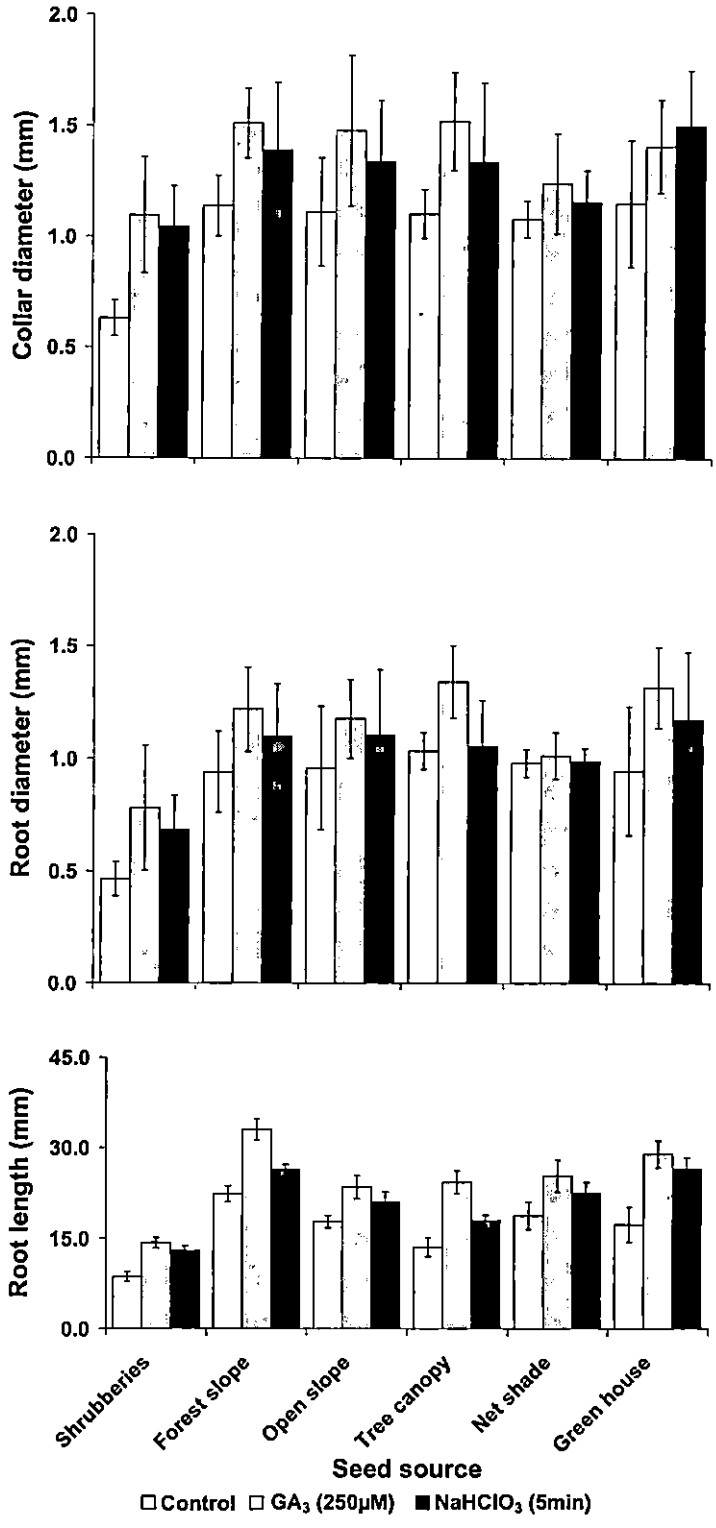

Figure 40. Effect of pre sowing chemical treatments on collar diameter, root diameter, root length in *Swertia chirayita* (error bars: standard deviations)

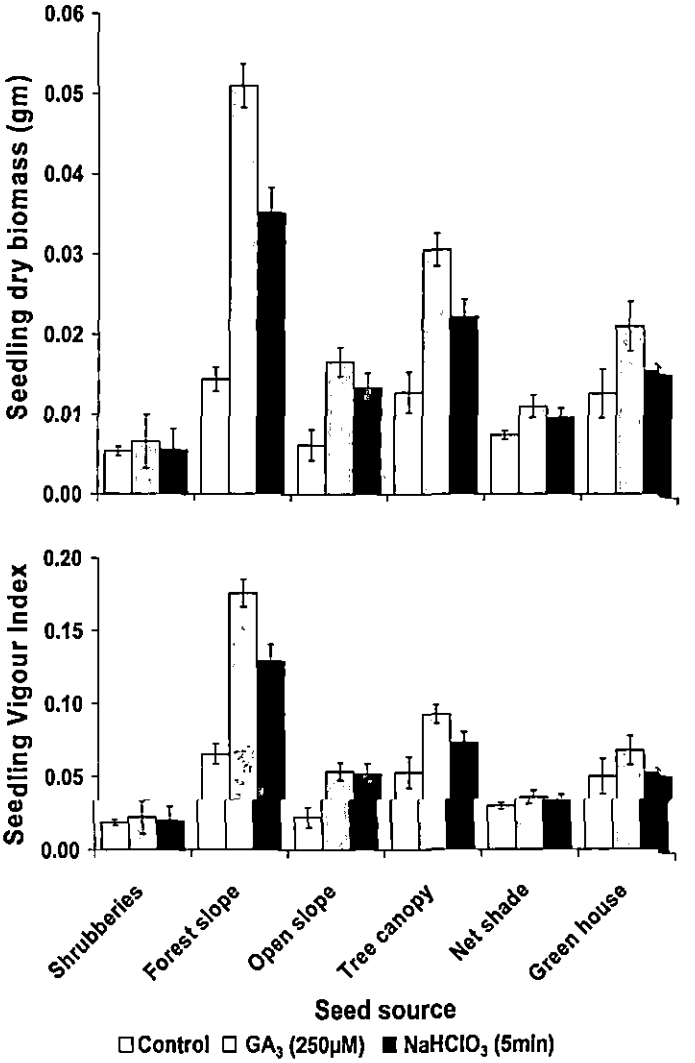

Figure 41. Effect of pre sowing chemical treatments on seedling dry biomass and seedling vigour in *Swertia chirayita* (error bars: standard deviations)