Chapter II

Induced Water Stress Experiment to Study the Contrasting Behaviour of TV23 and S.3A/3 Towards Drought Tolerance
2.1 Introduction:

Tea plant being perennial is subjected to a number of biotic and abiotic stresses throughout the year. Drought is one of the major abiotic stresses in 35% – 40% of the tea growing areas of North-East India (Singh and Handique, 1993) causing substantial yield losses up to 40% (Barua 1989; Jain 1999). Being a rain fed species, yield and quality of tea is highly dependent on availability of rain water. Although monsoon rains bring sufficient amount of rainfall, prolong dry spell within a year causes water stress on tea plants mainly during the months of December to March. Last year, “The Telegraph”, a national daily of India (Thursday, 18th Feb, 2010), has reported drying up of large numbers of tea bushes during the dry spell forcing the planters to carry out re-plantations in several districts of Assam. Recently, Tea Board of Kenya, world’s biggest exporter of black variety of leaves, has reported a decline of 16% in the first half of the year 2011. This decline in production has been largely attributed to hot and dry weather conditions experienced during the first quarter of the year as well as depressed and poorly distributed rainfall pattern experienced in most tea growing areas. Therefore, the present need of the tea industry is genetically improved superior planting materials tolerant to drought. Thus, it has become important to identify tea cultivars having high drought tolerance that can withstand and perform well under water stress environment.

The common parameters used by the plant physiologist to assess plant drought tolerance are leaf water potential (Eknayake et al., 1985; O’Toole and Moya, 1978; Bashar et al., 1990), water use efficiency (Donavan and Ehleringer, 1994; Dudley, 1996; Heschel et al., 2002), photosynthesis and transpiration rate, stomatal conductance and relative leaf water content (Bota et al., 2004; Damayanthi et al., 2010).

Water stress brings about many changes in plants. Among different physicochemical parameters leaf water potential, photosynthesis rate, transpiration rate, relative leaf water content, soil moisture and water use efficiency were found to have strong correlation with water stress tolerance in Tea (Singh and Handique, 1993). Besides, drought susceptibility index,
stomatal density, total soluble sugar content has also been successfully used in screening drought tolerant tea cultivars (Damayanthi et al., 2010). These physicochemical parameters were used in the present study to evaluate the drought tolerance of two tea cultivars.

Drought tolerance of plant is a complex trait often manifested by its physiological and biochemical reactions involving interplay of a vast array of genes and can be the basis for screening and selection of tea cultivars tolerant to water stress. To understand the molecular basis of drought tolerance, it is necessary to explore the vast array of gene expression pattern in different tea cultivars with contrasting drought tolerance. Therefore, it is important to differentiate the transcriptome of a drought tolerant tea plant from a susceptible one to know the basis of drought tolerance at molecular level. Comparing the gene expression pattern of a drought tolerant tea plant with a susceptible one is certainly a strategy to study gene expression pattern that may be responsible for contributing towards drought tolerance. To perform such a comparative analysis for identification of genes responsible for drought tolerance or susceptibility one must have tea plants with contrasting drought tolerance. In the present study we have selected two tea cultivars based on their contrasting drought tolerance in field condition.

2.2 Materials and Methods

2.2.1 Selection of Plant Materials

From 1949 to till date, Tocklai Experimental Station, Jorhat, Assam, has released a total of 31 tea clones (designated as TV1 to TV31). These clones has been vegetatively propagated by cuttings and well maintained in the experimental garden. Besides, there are a large number of garden series clones with good quality and yield that are popular among tea growers in North-East India as well as in other countries like Sri Lanka and Kenya (Bezbaruah and Dutta, 1977). All these clones have been characterised for quality, yield and tolerance to drought and other biotic and abiotic stresses based on different physiological, biochemical and morphological criteria (Singh et al., 1993). These are divided into three categories (Barbora et al.,
1996), viz., standard (above average yield and quality with yield potential of 3000 - 3500 kg MTPH), quality (high quality but average yield with yield potential of 2500 - 2800 kg MTPH) and yield (average quality but high yield with yield potential of 4000 kg MTPH or above). Among TV (Tocklai variety) clones TV1, TV9, TV19, TV23, and TV26 and among garden series clones P463, T.3E/3, S.3A/3, Tinali 17, BJ2, are very popular among tea growers for its higher yield and quality. These two groups of clones have been evaluated throughout the year in Tocklai Experimental Station under field conditions for drought tolerance (data not shown). TV23 was found to be highly tolerant among the TV clones while S.3A/3 was highly susceptible among the garden series clones. Moreover, TV23 has also been reported to have high yield and average quality (Barbora et. al., 1996) and is also one of the most preferred clones by the present day tea growers in North-East India. These two tea cultivars (TV23 and S.3A/3) having contrasting drought tolerance were used in the present study. An induced water stress experiment was designed to further substantiate their contrasting drought tolerance under controlled environmental variables as given below.

2.2.2 Induced Water Stress Experiment

2.2.2.1 Establishment of Tea Cultivars in Green House

Two years old sleeve grown healthy cultivars of TV23 and S.3A/3, raised from cuttings, were collected from the nursery of Tocklai Experimental Station (Longitude 94°12’E, Latitude 26°47’N and Elevation 96.5 amsl), TRA, Jorhat and potted in earthen pots (height 36 cm, bottom diameter 22 cm and top diameter 35 cm) containing sandy-loam soil (pH 4.8 – 5.1, bulk density 1.3 – 1.4 gm/cc, single super phosphate 500gm/cubic meter of soil) and adequate drainage at the base. Plants were kept in green house for 50 days with regular watering for complete establishment. All plants, except a few, were found to be well established with uniform growth and healthy phenotypes and considered for the induced water stress experiment.
2.2.2.2 Water Stress Treatment

Two sets of 50 days greenhouse grown healthy plants of each cultivar were taken as experimental and control. Each set contains 16 subsets of three plants each. The sixteen subsets for TV23 were designated as TV23T1 to TV23T16 for experimental plants and TV23C1 to TV23C16 for control.

Figure 2.1 Lay out of the induce water stress experiment.

- Photosynthesis rate
- Transpiration rate
- Leaf water potential
- Soil water content
- Water use efficiency
- Stomatal conductance

Parameters measured (From 12th day onward)

- Relative water content measurement (RWC)

Leaf sample collection

Stored at -80 °C for RNA isolation for SSH library construction and gene expression studies.

Plant Death (TV23 and S.3A/3)

28 days for TV23
22 days for S.3A/3

Figure 2.1 Lay out of the induce water stress experiment.
Similarly for S.3A/3, the designation was S.3A/3S1 to S.3A/3S16 for experimental and S.3A/3C1 to S.3A/3C16 for control plants. Therefore, a total of 96 (48 experimental and 48 control) plants of each cultivar were taken for the experiment. Drought was induced by withholding water supply in the experimental plants while control plants were regularly watered. The layout of the experiment and measurement of different physiological parameters is schematically represented in Figure 2.1

2.2.2.2.1 Measurement of Physiological Parameters and Sample Collection

The degree of water stress was monitored by measuring different physiological parameters of each subset (measured in 9 leaves/3 plants/subset) for both control and experimental plants from the 12th days of drought induction and continued till plant death. The measurement of following physiological parameters in fully expanded 1st and 2nd leaf from the shoot tip and collection of leaf samples (1st and 2nd leaf along with a bud) were carried out between 10.30 am to 11.30 am.

2.2.2.2.1.1 Leaf Water Potential ($\Psi_L$)

Leaf water potential was measured using a portable plant water status console (Soil Moisture Equipment Corporation, USA, Figure 2.4A), according to the technique described by Scholander et al. (1965). Prior to excising the leaf a small thin polythene bag was placed securely over the leaf to suppress transpiration. The leaf petiole was then cut with a sharp razor blade, leaving sufficient length of petiole to extend through the sealing stopper in the pressure chamber cover. After excision the leaf along with the polythene bag still secured around it was put immediately into the pressure chamber. This was done by placing the petiole through the cover with the cut edge facing the top-outside surface. The cover is then tightened around the petiole enough to assure that no gas will escape during the pressurization. Care was taken not to over-tighten as the internal structure of the petiole will be compromised and the subsequent pressure indication will be wrong. With the chamber lid in place, all that was visible from the top of the chamber was the flat cut edge of the petiole. After sealing the
chamber the gas was released very slowly by opening the compressed nitrogen valve. As the pressure in the chamber equals the pressure, or tension in the leaf, the xylem sap was forced out of the cut petiole surface. It’s very important to watch the cut surface of the sample for the appearance of the liquid which indicates that the leaf water potential has been reached. Use of a magnifying glass was found helpful to see the first bubble of sap on the cut edge of the petiole. As soon as sap started to push out the cut end of the petiole, valve was closed to stop any further increase in pressure. The corresponding pressure was recorded from the pressure gauge. This is the leaf water potential measured in negative (-) Bars. After this the pressure was released completely and the machine was ready for next measurement.

2.2.2.2.1.2 Relative Water Content of Leaf (RWC)

To measure the RWC, leaf was excised from the plant and immediately punched into a number of circles of 2.5 cm diameter, keeping the midrib at the centre, and fresh weight (FW) was taken. The cut leaf circles were then placed in distilled water and kept in refrigerator to become fully turgid. The time taken to become fully turgid was determined by repeated weighing of leaf circle until there was no further increase in weight. The fully turgid leaf circles were then blotted to remove surface moisture and turgid weight (TW) was taken. The leaf circles were then immediately incubated at 70°C for 48 hours followed by taking dry weight (DW). The RWC was calculated according to the following formula (Barrs, 1968).

\[
RWC\,\% = \left[\frac{(FW - DW)}{(TW - DW)}\right] \times 100
\]

2.2.2.2.1.3 Soil Water Content (SWC)

The soil sample was collected from a depth of 20 cm from the surface and immediately put in to a pre-weighted air tight container, weighed (FW), and put in oven at 100°C for 24 hours followed by reweighing (DW). The soil water content was calculated using the following formula.

\[
SWC\,\% = \left[\frac{FW - DW}{FW}\right] \times 100
\]
2.2.2.2.1.4 Gas Exchange Parameters

The rate of photosynthesis (Pn), transpiration (Tr) and stomatal conductance (Gs) were measured using an Infra Red Gas Analyzer (PP system: CIRAS-I; Figure 2.5B) according to the procedure described in the user manual.

2.2.2.2.1.5 Water Use Efficiency (WUE)

The water use efficiency was calculated at different stages of progressive drought using data obtained from the Infra Red Gas Analyzer (PP system: CIRAS-I) as described by Wibbe and Blanke (1995).

2.2.2.2 Sample Collection

After 12 days of drought induction we have started collecting physiological data and leaf samples for experimental and control plants for both the cultivars. Collected leaves were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation. These leaf samples were used for subtractive library construction as described in Chapter III.

2.3 Results

Different physiological parameters measured during progressive drought are given in Appendix 2.1 to 2.4. Analysis has shown significant variations of the parameters between drought induced and control plants in both the cultivars with the progression of drought. Therefore, these parameters are highly influenced by water stress. All the parameters measured has shown a decreasing trend with the progressive drought compared to the control plants. These parameters were found to be decreased under water stress in both the cultivars, although there were significant variations observed at a given water stress. The RWC of leaf was found to be decreased in both the cultivars with progression of drought and became zero on 19th day in S.3A/3 and 26th day on TV23 respectively (Figure 2.2c). Similarly, photosynthesis rate, stomatal conductance and transpiration rate were also found to be decreased rapidly in S.3A/3 compared to TV23 (Figure 2.2b, 2.2e and 2.2f) with the progression of drought at a given water stress and soil moisture level. As evident from the Figure 2.2a to 2.2g, TV23 was found to be more efficient in maintaining most of the measured physiological parameters at a
higher level of drought stress and for a prolonged period (up to 24 days) compared to S.3A/3 (17 days). The leaf water potential (Figure 2.1a) was found to be higher in S.3A/3 (high negative value) compared to TV23 at all the soil moisture levels. Therefore, TV23 is able to maintain a higher water status under severe drought compared to S.3A/3. On 18th day, most of the physiological activities (Pn, Gs, Tr) were found to be ceased in S.3A/3 (Figure 2.2b, 2.2e, 2.2f). Whereas, TV23 was able to maintain significant physiological activities even after 18 days of drought. However, most of the physiological parameters (Pn, Gs, Tr and WUE) became or approached zero in TV23 on 25th day of water stress. We consider these time points (18th day for S.3A/3 and 25th day for TV23) as wilting stage of the plants.

![Figure 2.2a Comparison of leaf water potential](image1)

![Figure 2.2b Comparison of rate of photosynthesis](image2)
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Figure 2.2c Comparison of relative water content

Figure 2.2d Comparison of soil water content

Figure 2.2e Comparison of stomatal conductance

Figure 2.2f Comparison of rate of transpiration
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Figure 2.2g Comparison of water use efficiency

Figure 2.3 Three stages of induced water stress

TV23

Before Wilting Stage (BWS)

Wilting Stage (WS)

After Wilting Stage (AWS)

S.3A/3

15th day

18th day

20th day
2.4 Discussion

In the present study we have used two tea cultivars having contrasting drought tolerance in field condition from a collection of well characterized tea germplasms from the experimental garden of Tocklai Experimental Station. These two cultivars were selected with the objective to validate their contrasting drought tolerance behaviour and to establish their suitability as plant materials for identification of drought responsive genes by comparative transcript profiling that may involve in drought tolerance or susceptibility in tea. To further substantiate their contrasting field drought tolerance behaviour, an induced water stress experiment was carried out in green house to investigate their intrinsic variation in drought tolerance based on different physiological parameters.

The contrasting drought tolerance of the two cultivars were indicated by observed drooping of leaf after 11 days and 20 days of water stress in S.3A/3 and TV23 respectively. As expected, both the cultivars showed contrasting differences in most of the physiological parameters scored (as shown in Figures 2.2 a–2.2g).

Water use efficiency of S.3A/3 was found to be higher than TV23 under well watered condition (Appendix 2.1 and 2.2). S.3A/3 was able to maintain...
higher WUE compared to TV23 up to 15 days of drought. However, with the progression of drought, S.3A/3 was unable to maintain WUE whereas TV23 has shown WUE up to 24 days of water stress. This indicated that after 24 days of water stress TV23 was able to maintain its photosynthetic activity and hence the WUE. This ability of TV23 to maintain WUE to a prolonged period under water deficit condition compared to S.3A/3 can be correlated with its higher drought tolerance as reported in other plants (Donovan and Ehleringer, 1994; Dudley, 1996; Heschel et al., 2002). Leaf water potential is one of the important quantitative parameter for measuring drought tolerance in crop plants (Bashar et al., 1990). Being a reliable indicator of plant water status (Kramer and Kozlowski 1983, Larcher, 1995), $\Psi_L$ was used in the present study to assess the water status of both the cultivars during progressive drought. Measurement of $\Psi_L$ has shown continuous increase under progressive drought in both the cultivars (Figure 2.2a). However, the increase of $\Psi_L$ is much more in S.3A/3 compared to TV23 at a given water stress. This indicates that TV23 was able to maintain a higher leaf water status compared to S.3A/3 that may be responsible for its contrasting drought tolerance. The tissues that can maintain high RWC and low $\Psi_L$ are reported to have high tolerance to drought (Aminzadeh and Eshghi, 2006; Ferrat and Lovatt, 1999; Khan and Stoddard, 2005). There was no substantial difference in RWC of leaf in both the cultivars on 13th day of water stress. However, its value decreased and became zero on 19th day in S.3A/3. Significantly, TV23 was able to maintain a relatively high RWC even after 25 days of water stress (Figure 2.2c). This means that TV23 is more efficient in holding water compared to S.3A/3 under same level of drought and therefore can maintain higher leaf water status. There were considerable variations observed in Gs in both the cultivars (Figure 2.2e). TV23 has shown lower Gs compared to S.3A/3 up to 17 days of water stress. This low value of Gs may be contributing for its higher drought tolerance as reported in other plants (Khan et al., 2007). However, the Gs became zero in S.3A/3 after 17 days of water stress, whereas TV23 was able to maintain it up to 23 days. Therefore, the lower Gs of TV23 compared to S.3A/3 may be contributing for its high drought tolerance.
From the above discussion it has become clear that TV23 is more efficient in maintaining higher water status compared to S.3A/3 at a particular level of water stress as indicted by $\Psi_L$ (lower negative value), low values of Gs and high values of RWC of leaf. Most of the physiological parameters (Pn, Gs, Tr and WUE) became zero after 17 days of water stress in S.3A.3 at soil water content of 6% (Appendix 2.2), whereas, TV23 was able to maintain some activities at that water stress (Appendix 2.1). We consider this point (18th day) as the wilting stage (WS) of S.3A/3 and designated as S.3A/3WS. This time difference to reach wilting stage (18th day) has clearly indicates that S.3A/3 is more susceptible to water stress than TV23 (25th day). We consider other two time points for S.3A/3, one two days before (i.e., 15th day) and the other one day after (i.e., 20th day) the WS and designated as before wilting stage (S.3A/3BWS) and after wilting stage (S.3A/3AWS) respectively (indicated by bold and underline in Appendix 2.2). Likewise, we have identified three stages (22th day, 25th day and 27th day) in TV23 and designated as TV23BWS (before wilting stage), TV23WS (wilting stage) and TV23AWS (after wilting stage) respectively (indicated as bold underline in Appendix 2.1). The changes in morphology of both the cultivars at these three stages of drought were shown in Figure 2.3. Samples collected from these stages of both the cultivars were used in the subsequent studies (Chapter III, IV and V).

2.5 Conclusion

Comparative analysis of different physiological parameters of the two cultivars under induced water stress has clearly indicated higher drought tolerance of TV23 compared to S.3A/3 which is also consistent with the field observation. Therefore, these two cultivars showing contrasting drought tolerance in the present study as well as in field conditions are the suitable material to study the basis of drought tolerance in tea at transcriptome level, as exemplified by many recent studies in other plants (Guo et al., 2009; Lenka et al., 2010; Jain and Chattopadhyay, 2010; Deokar et al., 2011). Accordingly, samples collected from these two cultivars at two stages of water stress (BWS and WS) have been used to identify and study the
differentially expressed drought responsive transcripts of tea under water stress in the subsequent Chapters.

References


