CHAPTER-IV

DISCUSSION

The objectives of the present study are, 1) Selection of related wild and cultivated species responding differently to water stresses, both, *in situ* and *ex situ.* 2) Analyzing phenotypic variations in wild and cultivated Convolvulaceae species towards water stress. 3) Analyzing physiological and biochemical alterations in these species exposed to water deficit. 4) Analyzing miRNA expression in these species in response to water deficit. All the results acquired from the study are discussed under separate headings with relevant literature supporting the observations. The conclusions of the study are derived with a holistic approach.

4.1. *In situ* soil water status and its impact on vegetation characteristics

Soil water content at experimental site was lower as compared to control site whereas other factors (such as soil physical properties, soil particle size, N status, topography, temperature, humidity, sunlight) were nearly similar at the two sites. The vegetation composition observed at these sites was similar, which was comparable to those reported previously by Dave and Krishnayya (2004). Species richness of herb and shrub species was not observed to vary between the two sites, indicating no major effect of water deficit on species richness. However, the density of plants was lower at experimental site than control, indicating the effect of water stress prevailing at the experimental site. Several previous reports showed an association between plant species density and water availability (Walter, 1971; Sharitz and McCormick, 1973; Wright, 1992). Density of *Acacia karroo* shrubs was observed to be adversely affected by soil water stress (O'Connor, 1995). Similar influence of soil water deficit was reported in herbaceous vegetation (Poulsen, 1996). Recently, Yahdjian and Sala (2006) and Atteya (2013) reported a significant decrease in plant density under water-deficit conditions. These reports support the findings of the current study. Apart from this, variations in plant morphological traits were studied by analyzing the height, stem diameter, and leaf/leaflet area of plants growing at two sites. Height and stem diameter of shrubs and herbs was lower under water deficit prevailing at experimental site which was in accordance to several previous findings (Prasad et al. 1982; Kirnak et al, 2001; Bakhshy et al., 2013). Several herb and shrub plant species such as *Artemisia, Ipomoea cairica, Calotropis procera, Catharanthus roseus*, *Impatiens walleriana*, *Petunia hybrida* and *Pelargonium hortorum* showed negative effects of water stress on their height (Carvalho et al., 2003; Tognon et al., 2012; Jaleel et al.,

2008; Boutraa, 2010; Chylinski et al. 2007; Andersson, 2011) indicating reduced plant growth under the influence of water deficit. Such reduction in plant height and growth is largely attributed to impaired mitosis, cell elongation and expansion (Farooq et al., 2009; Davatgar et al., 2009). Similar explanation can be given to the changes observed in this study. Studying variations in stem diameter under water stress was previously restricted to tree species (Chandrashekar et al., 1998; Devakumar et al., 1999). However, these studies were found equally promising for herb and shrub species (Aina et al., 2007; Kulkarni and Deshpande, 2007; Hanssens et al., 2012), which was also observed in the current study. Water deficit at experimental site was found to decrease stem diameter of both herbs and shrubs. Drought exposed *Salvia splendens (*herbaceous) and *Cassava* (shrub) showed similar effect on their stem diameter (Burnett et al., 2005; Aina et al., 2007). Decrease in stem diameter acts as an indicator of plant growth reduction under water deficit (Miralles-Crespo et al., 2010; Niu and Rodriguez, 2008). Another important morphological parameter controlling plant growth is leaf area. In the present study, the leaf area under water stress was lower in the herb and shrub species than compared to control. Similar findings were previously reported in *Q. acutissima, Z. mays, Populus x canadensis* clones, *Amaranthus* species, *Vigna unguiculata* and *Trifollium alexandrinum* (Traore et al., 2000; Xu et al., 2008; Marron et al., 2003; Liu and Stutzel, 2004; Anyia and Herzog, 2004; Lazaridou and Koutroubas, 2004). Reduction in leaf area affects the surface interaction with solar radiations causing decrease in photosynthesis (Earl and Davis, 2003; Xu and Zhou, 2008). Variations in all these parameters indicate that the general vegetation including both herbs and shrubs are affected by the water stress at experimental site.

4.2. Leaf morphometric charateristics of screened plants under *in situ* conditions

Analysis of leaf morphometric characteristics is important as it provides insights into plants ability to change leaf structure to cope with environmental variations (Backhaus et al., 2010). In current study, the screened plant species (i.e. six wild and six cultivated species, each belonging to six different families) showed considerable variations in leaf morphometric characteristics (such as leaf perimeter, leaf area, leaf length, leaf width, SLA and LDMC) at experimental site than compared to control site. Leaf perimeter (margin length along the leaf lamina) is a key region of the leaf,

as important events related to patterning, signalling and morphogenesis occur at the margins (Scarpella et al., 2006; Reinhardt et al., 2007). Changes in leaf perimeter can deform leaf shape and may influence its ability to withstand environmental stress (Royer et al., 2008). In response to water stress, occurring at experimental site, leaf perimeter of screened plant species decreased. Similar change in leaf perimeter under water deficit conditions was seen in jujube (*Ziziphus jujuba*) varieties (Li et al., 2015). Even leaves of *Ranunculus acris* growing at relatively semi-dry site were reported to have lower leaf perimeter than compared to wet site (Kołodziejek and Michlewska, 2015). Observations of the current study are in agreement with these reports. Leaf area also showed a reduction. Studies on *Q. acutissima, Manihot esculenta* (*Cassava*), maize and jujube varieties showed similar effect of water deficit on their leaf area (Xu et al., 2008; Traore et al., 2000; Alves and Setter, 2004; Li et al., 2015). Such decrease in leaf area can be explained by the decline in cell expansion and division (Hsiao *et al.* 1985, Niinemets and Kull 1998; Alves and Setter, 2004). In the current study, reduction in leaf area was relatively higher in cultivated species. In drought sensitive *Quercus ilex* and drought tolerant *Phillyrea latifolia*, the decrease in leaf area in response to water deficit was stronger in the drought sensitive species (Ogaya and Penuelas, 2006). In similar lines, Ober and Luterbacher (2002) and Farooq et al. (2010) even reported that genotypes of rice and sugar beet respectively having higher functional leaf area were more tolerant to drought than others. It indicates that drought sensitivity of a species may be associated to major reduction in leaf area. Moreover, the decrease in leaf area reduces the surface area for photosynthesis, thereby influencing plant growth rate and biomass production (Pereira and Chaves, 1993 and Xu and Zhou, 2008). Similar inferences can be made from the results of this study. Alterations in leaf area can be due to the changes in either or both of the leaf dimensions i.e. length and width (Gonzalez et al., 2010). In the present study, the alterations in these two leaf dimensions showed how decrease in leaf area is regulated under water deficit. The decrease in leaf length was almost similar in wild and cultivated species under stress but the decrease in leaf width was prominent in cultivated species than compared to wilds. Negative effects of water stress on leaf length and leaf width were earlier found in *Oryza sativa, Quercus acutissima*, *Leucadendron laxum*, *Leucospermum* species and *Protea eximia* (Chutia and Borah, 2012; Xu et al., 2009; Yates et al., 2010). Drought induced reduction in leaf length is

associated with inhibition of leaf elongation rate which can partly increase duration of leaf growth period (Avramova et al., 2015). *Nerium oleander* cultivers exposed to water stress even showed reduction in leaf length and width, wherein the decrease in both these parameters was relatively prominent in the drought sensitive cultivars (Hardy Pink and Hardy Red) compared to the tolerant ones (EP2 and EP1) (Niu and Rodriguez, 2008). Prominent decrease in leaf width amongst the cultivated species is comparable to those seen in sensitive cultivars of *Nerium* oleander (Niu and Rodriguez, 2008).

SLA, an important indicator of resource-use strategy, was found reduced in both wild and cultivated species in response to water deficit. This is reported by several previous studies (Gibson et al. 1991; Van Hees, 1997; Li et al., 2000). Similar findings were even reported in *Arachis hypogaea*, *Populus davidiana, Medicago falcata* and several Mediterranean species exposed to drought stress (Valladares and Sanchez-Gomez, 2006; Zhang et al., 2004; Painawadee et al., 2009; Wang et al., 2012). However in the current study, the reduction in SLA under stress was comparatively prominent in cultivated species than wilds. Corresponding to these findings, drought mediated differential reduction in SLA was reported in *Amaranthus* genotypes, which was associated with drought adaptation strategy of the genotype (Liu and Stutzel, 2004). As decrease in SLA under stress corresponds with relatively high investments in leaf defenses (Cornelissen et al., 2003), it may indicate species specific strategy of cultivated plants to invest more than wilds in order to combat stress. Reduction in SLA can affect the potential relative growth rate and net photosynthetic capacity of plants (Reich et al., 2002; Cornelissen et al., 2003; Liu and Stutzel, 2004). Relatively conspicuous decrease in the SLA of cultivated species may indicate its relatively higher sensitivity than wilds towards water stress. Contrary to the response of SLA, LDMC showed increase in response to water deficit in both the wild and cultivated species by almost similar levels. Previous studies on drought tolerant (C76-16, A-104, 08T-12), moderately tolerant (GG) and susceptible (AP-30) cultivars of Peanut showed that the tolerant cultivars possessed high LDMC and low SLA under drought than the other three genotypes (Dang et al., 2013). Similarly, drought tolerant (IL 9-2-5) and sensitive (M82) genotypes of *Solanum pennellii* were reported to show significant increase in LDMC under drought stress, with the tolerant one maintaining a higher value than compared to sensitive genotype (Rigano et al.,

2016). Results of the current study are in support with these findings. Further, as LDMC is related to the nutrient retention and is negatively correlated with the potential relative growth rate, increased LDMC in both the wild and cultivated species may indicate reduced growth rate and thereby resist drought (Ryser and Urbas, 2000; Cornelissen et al., 2003; Poorter and Garnier, 2007). Overall, leaf morphometric analysis suggests better tolerence of wild species as compared to cultivated species when exposed to water stress. Amongst these species, cultivated (*J. pentantha*) and wild species (*I. campanulata*) belonging to Convolvulaceae showed major variations in the studied leaf morphometric characteristics. Leaf perimeter, area, length, width and SLA showed larger reduction in *J. pentantha* than found in *I. campanulata.* As discussed above, the prominent reduction in these leaf morphometric traits can provide *I. campanulata* relatively higher drought tolerance than *J. pentantha*. Contrary to these morphometric characteristics, increase in LDMC was relatively higher in *I. campanulata* than *J. pentantha*. As discussed above, relatively more increase in LDMC in *I. campanulata* may impart better tolerance against water stress.

In addition to leaf traits, floral traits are even better known to show plastic response towards change in local environment (Holtsford and Ellstrand, 1992). In the present study, *I. campanulata* and *J. pentantha* showed variation in floral traits in response to water stress. Corolla size showed reduction in both the species by almost similar levels. Related to findings of current study, Carroll et al., (2001) found 33% reduction in corolla size, which was directly related to plant water status in *Epilobium angustifolium*. Herrera (2004) further supported this findings in *Rosmarinus officinalis* flowers under drought stress. Such reduction in corolla size is reported to be a mechanism to reduce water loss, as larger size of corolla incurs physiological cost owing to greater water uptake (Galen, 1999, 2000). In addition to corolla, reduction in pistil and stamen length was found in *I. campanulata* and *J. pentantha* under stress; wherein *J. pentantha* showed major reduction. Comparable to findings of present study, increased tolerance to stress was attributed to relatively lesser reduction in anthers and pistil (Suzuki, 1981; Suzuki, 1982; Hashimoto, 1961; Matsui and Omasa, 2002). However, Jagadish et al., (2010) reported unaltered pistil lengths in *O. sativa* in response to high temperature. The reduction in length of pistil and stamen can potentially affect the pollination process as stigma position is considered

to be important for successful pollination (Matsui and Kagata, 2003; Jagadish et al., 2010).

4.3. Phenotypic variations in *I. campanulata* and *J. pentantha* grown under *ex situ* water deficit

In response to *ex situ* water deficit, both the species show development of typical drought stress symptom such as wilting of leaves, however it was prominent in *J. pentantha* than *I. campanulata*. Several previous studies showed that under water deficit, wilting of leaves is indicative of the stress occurring in plants (Wu et al., 2009; Pellegrineschi et al., 2004; Yang et al., 2010). Previously, differential response to drought was reported in drought-tolerant (IT93K503-1) and drought-sensitive (CB46) cowpea genotypes, with sensitive once showing apparent symptoms of drought stress like leaf wilting (Barrera-Figueroa et al., 2011). Response of *J. pentantha* towards water stress is comparable to that of drought-sensitive cowpea genotype and indicative of the higher stress experienced by *J. pentantha* than *I. campanulata*. In addition to the physical symptoms, relative water content (RWC) and specific leaf area (SLA) were reduced in both the water stressed species than compared to control. Leaf RWC is considered as the best growth indices revealing the stress intensity (Alizade, 2002). RWC showed major reduction in *J. pentantha* under water deficit than *I. campanulata;* which is indicative of higher stress encountered in *J. pentantha*. Such low levels of leaf RWC can cause inhibition of photosynthetic activity (Bjorkman and Powles, 1984). Several previous studies in drought stressed bean leaves showed significant reduction in RWC, wherein drought tolerant bean cultivars showed capacity to maintain high RWC values under stress (Lazacano-Ferrat and Lovat, 1999; Ramos et al., 2003; Zlatev, 2005). Likewise, maintenance of high RWC under drought stress was found in drought tolerant wheat, *Astragalus gombiformis* and *Medicago sativa* (Schonfeld et al., 1988; Gorai et al., 2010). This was even found true in current study as high levels of RWC in *I. campanulata* aided in stress tolerance. Such tolerant plant species having the ability to maintain high RWC were associated with higher yields (Arjenaki et al., 2012). Further, as discussed above many reports show direct correlation between SLA and potential relative growth rate, and net photosynthetic capacity of a species (Cornelissen et al., 2003; Liu and Stutzel,

2004; Garnier et al., 2001). Similar effects of decrease in SLA (although very minor) are seen in present study.

4.4. Impact of *in situ* and *ex situ* water deficit on physiological and biochemical parameters of *I. campanulata* and *J. pentantha*

4.4.1. Impact on chlorophyll content

Leaf greenness under water stress is commonly affected by chlorophyll loss (Smirnoff, 1995; Sandoval-Villa et al., 2002). Under *in situ* conditions, chlorophyll levels were higher in both the species growing at control site than at experimental site. Previous studies analyzing the effect of infield water deficit on chlorophyll content of *Aegilops geniculata, Phlomis fructicosa*, *Rismarinus officinalis, Cicer arietinum, Vitis vinifera* and *Polygonum maritimum* showed similar findings (Zaharieva et al., 2001; Kyparissis et al., 1995; Munne-Bosch and Alegre, 2000 and Mafakheri et al., 2010; Maroco et al., 2002; Dinler and Aksoy, 2014). In the present study, the decline in chlorophyll levels was comparatively higher in water stressed *J. pentantha* than *I. campanulata*. Differential reduction in chlorophyll content between the plant species exposed to similar levels of drought stress has been previously reported (Jagtap et al., 1998; Parida et al., 2007; Guerfel et al., 2009). Drought resistant genotype of wheat (*Triticum aestivum*-Kavir) was reported to show relatively high levels of chlorophyll under stress than the susceptible genotype (Ghods and Tajan) (Arjenaki et al., 2012). Comparatively higher decline in the chlorophyll content of *J. pentantha* than *I. campanulata* exhibits its higher sensitivity to *in situ* water deficit. Similar to response observed *in situ*, chlorophyll content of both the species under *ex situ* conditions showed reduction under drought stress than compared to their respective controls.

Chlorophyll loss under *ex situ* drought conditions was previously reviewed and reported by several researchers (Anjum et al., 2011; Jaleel et al., 2009; Anjum et al., 2003; Surendar et al., 2013). Significant reduction in chlorophyll content in response to drought stress was also reported in *Sorghum bicolor, Catharanthus roseus, Gossipium hirsutum, Helianthus annuus* and *Cicer arietinum* (Jagtap et al., 1998; Jaleel et al., 2008; Massacci et al., 2008; Kiani et al., 2008; Mafakheri et al., 2010). Decline in chlorophyll levels were relatively conspicuous in *J. pentantha.* Cotton cultivars, varying in drought tolerance (GM 090304- drought tolerant and Ca/H 631- drought sensitive), showed varied chlorophyll degradation, with high degree of

degradation in the sensitive cultivar (Parida et al., 2007). Similar response is observed in the sensitive *J. pentantha.* As chlorophyll is an important pigment for photosynthesis, its decrease under drought stress can adversely affect the plant growth and development. Decrease in plant photosynthesis in response to declining levels of chlorophyll was previously validated by several researchers (Chaves et al., 2009; Flexas et al., 2004; Lawlor and Tezara, 2009). Mafakheri et al., (2010) reported a significant reduction in chlorophyll content, photosynthetic rate and plant growth (shoot length, number of pods and yield) in *Cicer arietinum* growing under in-field water deficit. It can be said that reduction seen in measured growth parameters are manifestations of loss in chlorophyll contents. Its manifestation was relatively lesser in tolerant-*I. campanulata*. The overall degree of chlorophyll reduction in both the species was comparatively higher under *in situ* conditions than *ex situ.* This could be due to variability in the duration of drought stress.

4.4.2. Impact on anthocyanin content

Anthocyanin levels are essential in analyzing sensitivity of plants towards stress (Yang et al., 2000; Gould, 2004). Levels of anthocyanin in *I. campanulata* and *J. pentantha* were higher at experimental site than at control site. Previous studies, analyzing anthocyanin concentration in *Vaccinium myrtillus* and *Eucalyptus grandis* subjected to in-field water deficit supported the findings of current study (Taulavuori et al., 2010; Coscolin et al., 2011). Excessive accumulation of anthocyanin can trigger formation of ROS and increase the heat load of the tissue resulting in photoinhibition (Hetherington, 1997; Ludlow & Bjorkman, 1984; Foyer et al., 1994; Steyn et al., 2002). The degree of anthocyanin accumulation was relatively higher in *J. pentantha* than *I. campanulata*. Differential degree of anthocyanin accumulation under the influence of drought was even found in other plant species (Efeoglu et al., 2009; Basu et al., 2010; Coscolin et al., 2011). Macar and Ekmekci, (2009) reported 4.4 and 3.1 fold rise in anthocyanin concentrations in Gokce and Canitez cultivars of *Cicer arietinum* respectively; wherein Canitez cultivars (with relatively lesser anthocyanin accumulation) showed better ability to overcome drought. Outcomes of the current study are in agreement to this report. Relatively higher accumulation of anthocyanin in *J. pentantha* than *I. campanulata* reveals its higher sensitivity towards *in situ* water stress.

Related to the response seen *in situ*, anthocyanin concentration under *ex situ* water deficit increased in both the species. However, the degree of increase was relatively higher in *J. pentantha* than *I. campanulata*. Drought induced anthocyanin accumulation was formerly seen in *Vigna unguiculata, Cucumis sativus* and *Gossypium herbaceum* (Balakumar et al., 1993; Yang et al., 2000; Deeba et al., 2012). Differential accretion of anthocyanin was found amongst the cultivars of *Solanum tuberosum* and *Zea maize* exposed to *ex situ* drought stress (Andre et al., 2009; Efeoglu et al., 2009). In three rice cultivars, exposed to *ex situ* drought, the maximum anthocyanin accumulation was reported in drought susceptible cultivar Pusa Basmati (PB) while the IR-29 and Pokkali (drought tolerant) cultivars showed relatively lesser accumulation (Basu et al., 2010). Response of sensitive *J. pentantha* under *ex situ* stress is comparable to that of susceptible rice cultivar. In general, degree of anthocyanin accumulation was higher under *in situ* stress as compared to the *ex situ* one. Pattern of variation seen in the two species is the same for both the stress conditions.

4.4.3. Impact on lipid peroxidation

Rise in levels of lipid peroxidation is regarded as an index of increasing oxidative stress (Meirs et al., 1992; Anjum et al., 2011). Levels of lipid peroxidation are measured as changes in malondialdehyde (MDA) content, a secondary end product of oxidative lipid degradation. In the current study, the MDA concentrations in both the species were higher at experimental site than at control site. Earlier studies on *Phillyrea angustifolia, Coffea* species (*C. arabica* and *C. liberica*) and *Boehmeria nivea* growing under *in situ* water deficit showed almost similar levels of increase in MDA content (Munne-Bosch and Penuelas, 2003; Cai et al., 2005; Huang et al., 2013). Bai et al., (2006) reported that when plants are subjected to drought, oxidative stress results in lipid peroxidation which indicates the possible damage to biological membrane. Increase in levels of lipid peroxidation in the two species under study, shows previlance of oxidative stress. Amongst these two species, the accumulation of MDA was higher in *J. pentantha* than *I. campanulata*. Previous studies amongst drought susceptible (HD-2329) and tolerant (C-306) wheat genotypes showed that the susceptible genotype had higher levels of lipid peroxidation in response to water stress (Sairam et al., 1998). Similar differential response towards water deficit was

shown by drought sensitive and tolerant cultivars of *Boehmeria nivea*, wherein the rise in MDA concentrations were dramatically high in the sensitive cultivar (46.8-67.8%) than the tolerant ones (36-36.2%) (Liu et al., 2005). Drought tolerant (Sardi 10, Tamantit and Rich 2) and sensitive (Ecotipo Siciliano) *Medicago sativa* cultivars, exposed to water stress, exhibited similar difference in MDA accumulation under stress (Slama et al., 2011). Outcomes of the current study move together with the findings of these reports. It suggest that stress endurance of *I. campanulata* can be related to lower levels of lipid peroxidation.

Similar to response noticed *in situ*, water deficit mediated surge in MDA content was found in the two species under *ex situ* conditions. It was relatively higher in *J. pentantha* than *I. campanulata.* In support with these findings, *Cicer arietinum* plants exposed to *ex situ* water deficit showed higher MDA accumulation than the well-water plants (Macar and Ekmekci, 2009). Likewise MDA accumulation was reported in several other plants such as *Helianthus annuus, Sorghum bicolor, Carthamus tinctorius* (safflower) and *Avena* species exposed to *ex situ* water deficit (Zhang and Kirkham, 1996; Pandey et al., 2010; Javed et al., 2013). Contrary to these findings, MDA concentration was not found to increase significantly in *Polygonum maritimum* under *ex situ* water stress (Dinler and Aksoy, 2014). This response can be due to superior stress tolerance of *P. maritimum* which may limit the formation of free radicals and thereby minimise MDA accumulation. Differential accumulation of MDA found in the two species under study is comparable to previous reports. For example, among two *Phaseolus vulgaris* species, drought-sensitive species showed higher levels of MDA than the drought-tolerant one (Turkan et al., 2005). Consistently low levels of lipid peroxidation were even observed in drought tolerant *Alternanthera philoxeroides* than moderately tolerant *Oryza sativa* (Gao et al., 2008). Amongst the two *Chrysanthemum* cultivars exposed to drought stress, the drought sensitive cultivar (Nannong Jingyan) showed 1.4-2.6 times increase in MDA levels while the drought tolerant cultivar (Nannong Xuefeng) showed minor change (Sun et al., 2013). It suggests that drought sensitive species accumulate more MDA, possibly due to higher production of ROS. Although MDA accumulation was observed in both the species growing under *in situ* and *ex situ* water deficit, it was comparatively higher under *ex situ* condition indicating occurrence of higher oxidative stress.

4.4.4. Impact on Superoxide dismutase (SOD)

Drought disrupts the equilibrium between the production and the scavenging of ROS leading to sudden increase in intracellular levels of ROS. This can affect cellular functioning by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation (Gill and Tuteja, 2010). Increased levels of ROS is counteracted by variety of antioxidants, amongst which SOD is the most effective antioxidant providing front-first line defense against accumulated ROS (Mittler, 2002; Cruz de Carvalho, 2008). In the current study, eight SOD isoforms were identified from both the species, which consist of two MnSODs, two FeSODs and four CuZnSODs. It showed that both the species possessed the known three basic type of SOD isoforoms (MnSOD, FeSOD and CuZnSOD) (Alscher et al., 2002). Previous studies on model plants *A. thaliana* and *Pisum sativum* identified seven (one MnSOD, three FeSODs and three CuZnSODs) and five SOD (one MnSOD, two FeSODs and two CuZnSODs) isoforms respectively (Kliebenstein et al., 1998; Iturbe-Ormaetxe et al., 1998). In *Glycyrrhiza uralensis*, a total of six SOD isoforms consisting of one MnSOD, one FeSOD and four CuZnSODs were identified (Pan et al., 2006). Huseynova et al., (2014) identified three MnSOD, one FeSOD and five CuZnSOD isoforms in *Triticum durum*. SOD isoforms identified in the current study are comparable to these reports and indicate that the number of SOD isoforms vary between the species. Several former studies support this finding (Pan and Yau 1992; Ormrod et al. 1995; Fernandez-Ocana et al. 2011). Under *in situ* water stress, both the species showed induction in the activity of all the SOD isoforms; which was very distinctly represented by CuZnSODs. However, amongst the two species, the increase in the activity of SOD isoforms was relatively higher in *I. campanulata* than *J. pentantha*. Similar finding were reported amongst the drought tolerant (*C. arabica*) and sensitive (*C. liberica*) coffee species growing under in-field water deficit, wherein the tolerant one showed relatively higher activity of SOD isoforms (Cai et al., 2005). Likewise, drought tolerant *Populus* clones (Dorskamp) showed relatively higher activity of SOD isoforms than the susceptible clone (Luisa Avanzo) exposed to water-deficit (Marron et al., 2006). Simova-Stoilova et al., (2009) reported relatively prominent increase in the activity of all SOD isoforms in two drought-tolerant (Yantar and Zlatitsa) wheat varieties than the sensitive ones (Miziya and Dobrudjanka), amongst which CuZnSODs showed distinct response in the tolerant varieties.

Relatively higher expression of SOD isoforms in *I. campanulata* than *J. pentantha* reveals its higher tolerance to *in situ* water stress. This role of SOD isoforms is confirmed by studies on transgenic plants overexpressing either of the SOD isoforms. For example, transgenic *Medicago sativa* overexpressing MnSODs tended to have higher tolerance towards water-deficit (McKersie et al., 1996; Samis et al., 2002). Transgenic *Nicotiana tabacum* plants overexpressing FeSODs showed higher tolerance to oxidative stress (Van Camp et al., 1996). Similarly, *Nicotiana tabacum* and *Capsicum annum* plants overexpressing CuZnSODs showed enhance tolerance to water stress (Badawi et al., 2004; Chatzidimitriadou et al., 2009). Relatively higher activity of SOD isoforms in *I. campanulata* under stress can regulate ROS which other wise can cause lipid peroxidation. This is confirmed by observed low levels of lipid peroxidation (as discussed previously) in *I. campanulata* .

Similar to the response observed *in situ*, both the species exposed to *ex situ* stress showed induction in the activity of SOD isoforms which was relatively higher in *I. campanulata* than *J. pentantha.* Increase in the activity of MnSODs (MnSODI and MnSODII) and FeSODs (FeSODI and FeSODII) was significant only in *I. campanulata*, while that of CuZnSODs was significant in both the species. Drought tolerant- *P. acutifolius* and sensitive- *P. vulgaris* subjected to *ex situ* water deficit showed relatively higher activity of all SOD isoforms in the tolerant one (Turkan et al., 2005). Czovek et al., (2006) reported *ex situ* drought medicated increase in activities of SOD isoforms in drought tolerant accessions of *Aegilops biuncialis* and *Triticum aestivum* (Ae550 and Sakha respectively) and no change in the sensitive accessions (Ae1050, Cappelle). In addition the author reported that amongst all the SOD isoforms the activities of CuZnSODs were dominant in *A. biuncialis* and *T. aestivum* accessions. Similar findings were reported in drought tolerant-*Alternanthera philoxeroides* and sensitive-*Oryza sativa* exposed to *ex situ* water deficit (Gao et al., 2008). Comparatively higher activity of SOD isoforms (specifically CuZnSOD) in *I. campanulata* can contribute for its higher tolerance towards *ex situ* water deficit. On the contrary, transient/less induction of *J. pentantha* SODs may demonstrate its sensitivity to stress. Although SOD isoforms of both the species showed increase in their activity in response to both *in situ* and *ex situ* water deficit; the degree of induction for each isoform was different. MnSODs and FeSODII showed significant increase in their activity in both the species under *in situ* conditions; however it was

not the case under *ex situ* conditions. On the contrary, significant increase in CuZnSODs was observed under both the conditions, which was relatively higher under *ex situ* conditions. These results indicate that activity of MnSOD and FeSOD isoforms show relatively higher activity under *in situ* stress while CuZnSODs show higher activity under *ex situ* stress in these species.

4.5. Small RNA populations of *I. campanulata* and *J. pentantha*

Small non-coding RNAs play crucial role in controlling gene expression under all conditions, including survival under water stress. Four to eight million total small RNA reads were identified by high throughput sequencing (HTP) from *ex situ* control and drought stressed libraries of *I. campanulata* and *J. pentantha*. Most of the previous studies used similar technique to identify several lakhs to million small RNAs from genome sequenced model and crop plant species such as *Arabidopsis thaliana*, *Oryza sativa*, *Triticum aestivum*, *Gossypium hirsutum*, *Glycine max*, *Medicago truncatula*, *Solanum lycopersicum* and *Populus balsamifera* exposed to unstressed and stressed conditions (Rajagopalan et al., 2006; Fahlgren et al., 2007; Barakat et al., 2007; Sunkar et al., 2008; Szittya et al., 2008; Subramanian et al., 2008; Moxon et al., 2008; Kwak et al., 2009; Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009; Xin et al., 2010; Wang et al., 2011; Eldem et al., 2012). However, very few studies identified several million small RNAs through HTP sequencing in non-model plants with no known genome information (Wang et al., 2012; Guzman et al., 2012; Paul et al., 2013). Results of the present investigation profiling small RNAs in non-model wild (*I. campanulata)* and cultivated (*J. pentantha)* plants are comparable to these reports. In addition, current study compares the drought mediated change in small RNA profiles in these two related species. Such comparative study is hardly seen in earlier reports indicative of the uniqueness of present study. This kind of studies are gaining importance in recent times as most of the illustrious functional role of small RNAs (specifically miRNAs) in plant biological processes mostly comes from genome sequenced plants and seldom non-model plants have been investigated (Unamba et al., 2015).

The total unique sequence reads identified from the control and drought stressed libraries of the two Convolvulaceae species ranged from one to four million. This read count was comparable to the total unique sequence reads identified from control

libraries of *Liriodendron chinense* and *Brassica oleracea*, plants lacking genome information (Wang et al., 2012; Lukasik et al., 2013). The size distribution of these total unique small RNA reads in the control and drought stress libraries of both the species was almost similar. In both the libraries highest reads was observed for 24-nt size class which was followed by second highest peak at 23-nt in control libraries and 21-nt in drought stress libraries. Similar to control libraries, abundance of small RNA reads was reported in the control samples of *Lagenaria siceraria, Cucurbita moschata, Cucurbita pepo*, *Citrullus lanatus, Ammopiptanthus mongolicus, G. hirsutum* and *Phaseolous vulgare* (Pelaez et al., 2012; Jagadeeswaran et al., 2012; Gao et al., 2016; Ruan et al., 2009). However control and drought libraries of *M. truncatula* and *Hordeum vulgare* showed small RNA abundances similar to drought stressed libraries of the two species under study (Wang et al. 2011; Hackenberg et al. 2015). Size of small RNAs is often a useful representative for biological function. Small RNAs belonging to 24-nt size class largely consist of endogenous siRNAs that are produced from transposons and DNA repeat sequences and usually associate with AGO4 regulating RNA-directed DNA methylation (Qi et al., 2006; Kasschau et al., 2007). While small RNAs that are 21-nt long mostly comprise miRNAs and tasiRNAs (trans-acting short interfering RNAs) (Montes et al. 2014). In *A. thaliana*, nearly 80% of miRNAs are 21-nt long (Liu et al., 2014). In control and drought libraries of both the species the abundance of this class (21-nt size) was highest amongst the unique miRNA reads. However amongst the two libraries, their abundance was prominently higher in drought libraries as compared to control ones, indicating accumulation of 21-nt unique miRNA reads in drought stress libraries.

Many of the identified plant miRNA families are deeply conserved in plant lineages indicating that they bear conserved biological function (Sunkar and Jagadeeswaran, 2008). This characteristic feature can act as a powerful tool to identify miRNAs for non-model plants (lacking genome information) through homology search (Weber, 2005; Zhang et al., 2006). Using this strategy in the current study, 213 and 177 miRNAs belonging to 41 conserved miRNA families were identified from control and drought stressed libraries of *I. campanulata* respectively. While from *J. pentantha*, 150 and 176 miRNAs belonging to 35 conserved miRNA families were identified from control and drought stressed libraries respectively. Previously, Paul et al., (2013) identified 53 miRNAs belonging to 26 conserved and less conserved miRNA families

in *Vigna mungo* (an important legume crop lacking genome sequence information), indicating that the miRNAs identified in the current study are considerably higher. Similar studies in *Eugenia uniflora and Brassica oleracea* (lacking genome sequence information), reported 204 and 261 conserved miRNAs represented by 45 and 62 miRNA families respectively (Guzman et al., 2012; Lukasik et al., 2013). Likewise, 142 and 496 miRNAs belonging to 42 and 97 conserved miRNA families were reported from *L. chinense* and *Paulownia fortunei* (both largely found restricted to China) respectively (Wang et al., 2012; Niu et al., 2014). While in Jute (*Corchorus olitorius* and *Corchorus capsularis*) missing native genome sequence, 164 miRNAs belonging to 23 conserved miRNA families were found (Islam et al., 2015). Recently, Gao et al., (2016) identified 100 miRNAs representing 35 conserved miRNA families in *A. mongolicus,* a relic of tertiary period that can grow in harsh environment. The number of conserved miRNAs identified from the two Convolvulaceae plants is comparable to those reported in these studies. Amongst the miRNA families identified in the two species under study, 34 families were analogous and remaining 8 were alien (i.e. identified in either of the species). Most of the analogous miRNA families, which includes miR156, miR157, miR159, miR162, miR166, miR168, miR172, miR319, miR394, miR396 showed high expression indicating their crucial role in normal development of both the species. These miRNAs were even reported to have important developmental role in other plant species. For instance, miR156 and miR172 regulate transition from juvenile to adult phase by targeting SPL (SQUAMOSA PROMOTER BINDING-LIKE) transcription factors; miR157/SPL controls floral organ development; miR168 regulates normal plant development by targeting AGO1; miR166/AGO10 maintains the shoot apical meristem; and miR319/TCPs (TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTORS) and miR396/GRFs (GROWTH-REGULATING FACTORS) affect cell division and regulate leaf morphogenesis (Palatnik et al., 2003; Vaucheret et al., 2004; Wu et al., 2009; Zhu et al., 2011; Wang et al., 2011; Liu et al., 2017). While among the alien miRNAs, expression of miR477 was specifically identified in wild species, *I. campanulata*. Similar to this finding, expression of miR477 was reported in leaves of wild progenitor of *Manihot esculenta* as compared to the cultivated ones (Chen et al. 2015). miR477 was observed to show interesting phylogenetic distribution, due to its presence in bryophytes, then absence in lycopods, gymnosperms, all surveyed

monocots and; presence in *Aquilegia coerulea* (an intermediate model species between monocots and core eudicots) and core eudicots (Axtell et al., 2007; Lu et al., 2007; Puzey and Kramer, 2009). Amongst the eudicots, its expression was previously reported in *Populus trichocarpa*, *Vitis vinifere* and *Nicotiana tabaccum* (Lu et al., 2005; Jaillon et al., 2007; Tang et al., 2012). miR477 in *P. trichocarpa*, is predicted to target a member of the GRAS gene family and a NAC-domain mRNA that are largely involved in developmental patterning (Lu et al., 2005; Laufs et al., 2004; Mallory et al., 2004). Species specific expression of miR477 in *I. campanulata* can be considered to perform similar function.

4.5.1. Innate variations in miRNA expression between *I. campanulata* and *J. pentantha*

Although most of the miRNAs identified in both the species overlapped, their levels of expression were observed to be different in both the species under control conditions. Expression of miR166, followed by miR396 was most abundant in the control samples of both plant species. Predominant abundance of miR166 was previously reported in *G. max, Sorghum bicolor* and *Cucurbit* species (Li et al., 2011; Zhang et al., 2011; Jagadeeswaran et al., 2012). However, miR166 showed almost two fold greater expression in *J. pentantha* than compared to *I. campanulata*. miR166 mediated regulation of HD-ZIP III transcription factor is highly conserved among plant lineage which is known to modulate diverse facets of plant development such as leaf polarity, xylem differentiation in root, and modulation of lateral root growth under drought stress (Jung and Park, 2007; Sakaguchi and Watanabe, 2012; Bakhshi et al., 2016). Differential expression levels of miR166 amongst the wild and cultivated species under study may suggest differential regulation of its target/s, impacting some of the targeted responses. Similarly, several other miRNA families such as miR156, miR160, miR164, miR167, miR172, miR319 and miR403 were more abundantly expressed in *I. campanulata*, while miR159, miR168 and miR408 were more abundantly expressed in *J. pentantha*. Most striking difference in miRNA abundances was seen for miR403, wherein *I. campanulata* showed almost 600 fold greater expression than *J. pentantha*. Both the species showed only two miR403 isoforms. miR403 is mostly found restricted to dicot families like Malvaceae, Vitaceae, Salicaceae and Solancaceae (Jagtap and Shivaprasad, 2014). miR403

mediated regulation of AGO2 was found to be conserved in *Salvia miltiorrhiza* and *A. thaliana* (Allen et al., 2005; Shao and Lu, 2013). In *S. lycopersicum*, miR403 was reported to function in plant development by affecting expression of AGO2, which was suspected to further alter miR168/AGO1 homeostasis (Zhang et al., 2015). Its innate high levels in *I. campanulata* may be indicative of its differential function role than *J. pentantha*. Similarly, miR858 showed relatively higher expression in *I. campanulata* than almost no expression detected in *J. pentantha.* miR858 mediated regulation of target MYBs (myeloblastosis) have been previously known in apple (*Malus* × *domestica*), *Vitis vinifera* and *Salvia miltiorrhiza* (Xia et al., 2012; Rock, 2013; Li and Lu, 2014). Guan et al., (2014) reported that in *A. thaliana*, miR858 mediated regulation of MYB2 functions in trichome fibre development. While in *S. lycopersicum* miR858-targeted MYB2 was reported to control anthocyanin biosynthesis (Jia et al., 2015). More recently miR858 was established to be light-regulated and function in flavonoid biosynthesis, growth and development of *A. thaliana* (Sharma et al., 2016). miR858 of *I. campanulata* can be thought to have similar functional role. On the other hand, expression levels of miR390 and miR398 was 30 and 40 fold greater in *J. pentantha* respectively, than compared to *I. campanulata*. miR390 modulates the production of tasiRNAs that targets AUXIN RESPONSE FACTOR (ARF) mRNAs that are crucial for auxin signaling. The miR390/tasiRNA/ARF regulatory system directs developmental transitions and organ polarity in flowering plants. This pathway is highly conserved in land plants (Xia et al., 2017). miR398 is another highly conserved miRNA that regulates four target mRNAs in *A. thaliana*, cytosolic COPPER/ZINC SUPEROXIDE DISMUTASE1 (CSD1), chloroplastic CSD2, a subunit of the mitochondrial cytochrome *c* oxidase (COX5b-1) and COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS1). It suggests crucial function of miR398 in regulating copper homeostasis. Relatively higher expression of miR390 and miR398 in *J. pentantha* may portray their distinctive role from *I. campanulata*. These mature miRNAs of both the Convolvulaceae species showed predominance of U at 5' end. It is a characteristic feature of mature plant miRNAs and is proposed to play an important role in miRNA biogenesis or RISC formation (Zhang et al., 2006; Fahlgren et al., 2010; Liu et al., 2011). C is the dominant nucleotide at position 18 and 19 in the two species under study, which was very similar to that observed in miRNAs of *N. benthamiana*, *G. max*

and other known plant species (Zhang et al., 2006; Liu et al., 2011; Yin et al., 2015). However dominance of U at position 2 in miRNAs of *I. campanulata* differed from that of *G. max* and other plant miRNAs (Zhang et al., 2006; Liu et al., 2011).

4.5.2. Impact of *in situ* and *ex situ* water deficit on miRNA expression of *I. campanulata* and *J. pentantha*

miRNA medicated plant response to water deficit have been widely studied previously (Sunkar and Zhu, 2004; Liu et al., 2008, Zhou et al., 2010; Trindade et al., 2010; Kantar et al., 2011), however, this study could be reporting for the first time about comparative analysis towards changes in miRNA profiles in wild-tolerant and cultivated-sensitive Convolvulaceae species. Under both *in situ* and *ex situ* water deficit, drought responsiveness of miRNAs varied between the two species. Under *ex situ* water stress, several miRNAs (miR156, miR159, miR160, miR164, miR167, miR171, miR172, miR393, miR394, miR395, miR396 and miR399) showed opposite regulation; wherein these miRNAs were downregulated in *I. campanulata* while upregulated in *J. pentantha*. This pattern was also confirmed by blot analysis. This shows differential mechanism of miRNA regulation in a drought tolerant (*I. campanulata*) and sensitive (*J. pentantha*) Convolvulaceae species in order to combat stress. While under *in situ* water deficit most of the analysed miRNAs in both the species were downregulated, which includes miR156, miR159, miR160, miR397, miR168, miR171, miR169, miR396, miR393 and miR167. This showed that expression pattern of most of the conserved miRNAs remained similar under *in situ* and *ex situ* stress in *I. campanulata*, which was not seen in *J. pentantha*. Targets of these miRNAs are also known to be highly conserved in different plant families (Floyd and Bowman, 2004; Zhang et al., 2006).

miR156 is the first documented and one of the most conserved plant miRNAs. miR156 in *I. campanulata* was downregulated under both *in situ* and *ex situ* drought conditions; while in *J. pentantha* it was downregulated under *in situ* stress and upregulated under *ex situ* stress. Expression of miR156 was upregulated in water stressed *A. thaliana*, *V. unguiculata*, *Phaseolus vulgaris* and *Brachypodium distachyon*; and downregulated in *Vitis vinifera*, *O. sativa*, *G. hirsutum* (Liu et al., 2008; Barrera-Figueroa et al., 2011; Zhou et al., 2010; Nageshbabu et al., 2013; Bertolini et al., 2013; Xie et al., 2015; Pagliarani et al., 2017). miR156 negatively

regulates the expression of SPL genes (Bertolini et al., 2013; Barrere-Figueroa et al., 2011). By regulating its target, miR156 modulates different aspects of development like vegetative phase change, plant height, leaf morphology, leaf trichome development, chlorophyll levels and anthocyanin biosynthesis (Gou et al., 2011; Feng et al., 2016; Shi and Xie, 2014; Yu et al., 2010; Wang et al., 2009; Wu et al., 2009). Indeed transgenic *N. tabaccum* plants having elevated and reduced levels of miR156 showed significant alterations in leaf shape and chlorophyll content as compared to wild plants (Feng et al., 2016). In the current study, both the two species showed leaf morphological variations in response to water stress. At physiological level, the two species showed reduction in chlorophyll content and induction in anthocyanin levels after exposure to drought. It suggests possible role of miR156 in modulating these traits in the two species under water deficit. miR156 regulates the transcription of miR172 through its effect on SPLs. In the current study, miR172 showed opposite regulation in the two species under both the drought conditions, wherein it was downregulated in *I. campanulata* and upregulated in *J. pentantha*. Previously, drought triggered upregulation of miR172 was reported in *A. thaliana*, *N. tabaccum* and *Eleusine coracona*; while its levels were downregulated in *G. hirsutum*, *O. sativa* and *P. vulgaris* (Liu et al., 2008; Xie et al., 2015; Farzier et al., 2011; Nageshbabu et al., 2013; Zhou et al., 2010; Nageshbabu and Jyothi, 2013). miR172 targets the transcription of AP2-like genes {such as TOE1, TOE2, TOE3, SCHLAFMUTZE (SMZ) and SCHNARCHZAPFEN (SNZ)} and thereby regulates flowering time (Aukerman and Sakai, 2003; Jung et al., 2007; Schmid et al., 2003). Overexpression of AP2-like genes in *Arabidposis* causes late flowering, whereas its loss-of-function leads to early flowering (Aukerman and Sakai, 2003). Late flowering plants withstand water stress by adopting drought avoidance strategy which allows the plants to acclimatize to water deficit over time and thus providing an advantage for fitness; while early flowering is considered as drought escape strategy (Schmalenbach et al., 2014). It indicates that miR172 might be able to contribute towards drought tolerance. Therefore expression pattern of miR172 in *I. campanulata* can be one of the possible reasons for its better drought tolerance.

miR396 is another well conserved and highly expressed miRNA that controls plant leaf growth. miR396 expression was reduced in drought tolerant- *I. campanulata* under both *in situ* and *ex situ* drought. Downregulation miR396 was also noticed in *in*

situ and *ex situ* stressed *J. pentantha* according to blot analysis. However sequencing results portrayed upregulation under *ex situ* drought. The lack of correlation between sequencing and northern blot results for miR396 expression in *ex situ* stressed *J. pentantha* could not be attributed to the source of RNA as same RNA samples were used for sequencing and Northern blot analysis. This lack of correlation between sequencing-based profiling and Northern profiling may largely be attributed to the biased-ligation with the adapters (Reddy et al., 2009) or sequencing problems. Drought triggered upregulation of miR396 was reported in *A. thaliana*, *N. tabaccum* and *B. distachyon* (Liu et al., 2008; Yang and Yu, 2009; Bertolini et al., 2013). Contrarily its downregulation was reported in *A. mongolicus, O. sativa* and *Vigna unguiculata* under drought (Gao et al., 2016; Zhou et al., 2010; Barrera-Figueroa et al., 2011). miR396 targets the expression of GRF genes (Jones-Rhoades and Bartel, 2004). Although downregulation of miR396 was seen in both the species, it was comparatively higher in *I. campanulata* which can lead to relatively higher expression of target genes. Strong expression of GRF indicates active growth and development of plant (Kim et al., 2003). It indicates that even under drought stress the lesser deviation from normal growth of the tolerant species *I. campanulata* might be attributed to expression pattern of miR396.

miR160 and miR167 are closely linked families that are considered indispensable for plant growth and development (Rhoades et al., 2002; Liu et al., 2010). Under *ex situ* drought, expression of miR160 and miR167 were downregulated in *I. campanulata* and upregulated in *J. pentantha*. While under *in situ* drought conditions their levels showed reduction in both the species. Previously miR160 was reported to show reduction in its expression in drought stressed *M. esculenta*, *P. trichocharpa* and *S. bicolor*; and accumulation in *Prunus persica* and *Saccharum* spp. (Ballen-Taborda et al., 2013; Shuai et al., 2013; Hamza et al., 2016; Eldem et al., 2012; Gentile et al., 2015). Downregulation of miR167 was reported in *A. thaliana*, *O. sativa*, *Helianthus annus*, *M. esculenta* and *Panicum virgatum* (Sun et al., 2012; Phookaew et al., 2014; Khakesifidi et al., 2015; Liu and Chen, 2009; Liu et al., 2008). miR160 regulates expression of ARF10, ARF16 and ARF17; and miR167 regulates expression of ARF6 and ARF8 (Turner et al., 2013; Gutierrez et al., 2009). ARF6, ARF8 and ARF17 have overlapping expression profiles during adventitious rooting and that they regulate each others expression by modulating the homeostasis of miR160 and miR167

(Gutierrez et al., 2009). As adventitious roots contribute significantly to soil moisture uptake under water deficit, miR160 and miR167 can be thought to play crucial role under drought. Downregulation of miR160 and miR167 was associated with drought tolerant variety of Sorghum (Hamza et al., 2016). Similar regulation of these two miRNAs was also seen in the drought tolerant *I. campanulata* which was found unaltered under *in situ* and *ex situ* drought conditions.

miR393 also controls Auxin homeostasis by targeting Auxin receptor TIR1 (transport inhibitor response 1) and TAS3-ARF (Windels and Vazquez, 2011; Chen et al., 2011). Under *ex situ* water stress, sequencing results showed very low expression of miR393 due to which it was not included as differentially regulated by drought (as per the predecided cut off criterion). However the small RNA blot analysis clearly revealed its induction by *ex situ* drought in *J. pentantha* but not in *I. campanulata*. While under *in situ* stress expression of miR393 was downregulated in both the species. Earlier studies on *A. thaliana*, *O. sativa*, *Saccharum* spp., *V. unguiculata* and *P. vulgaris* showed drought mediated accumulation of miR393 (Sunkar and Zhu, 2004; Zhao et al., 2007; Ferreira et al., 2012; Barrera-Figueroa et al., 2011; Arenas-Huertero et al., 2009). miR393 dynamics in *ex situ* drought stressed *J. pentantha* is comparable to these reports. Such dehydration triggered induction in the levels of miR393 can cease plant growth and development (Sunkar and Zhu, 2004; Ferreira et al., 2012). Whereas reduction in miR393 expression was associated with drought tolerance, as tolerant *G. max* genotype showed miR393 downregulation while the sensitive one showed upregulation (Kulcheski et al., 2011). Similar response of miR393 towards *in situ* and *ex situ* water deficit was seen in drought tolerant- *I. campanulata*.

The two closely related yet distinct miRNA families, miR319 and miR159 target TCP and MYB genes respectively (Reichel and Millar, 2015). miR319 was observed to accumulate in the two Convolvulaceae species in response to *ex situ* stress. However under *in situ* stress its levels were induced in *J. pentantha* and reduced in *I. campanulata*. Induction of miR319 in response to drought was reported in *A. thaliana*, *O. sativa*, *H. vulgare*, *Zea mays* and *B. distachyon* (Sunkar and Zhu, 2004; Zhou et al., 2010; Hackenberg et al., 2015; Wei et al., 2009; Bertolini et al., 2013). Whereas miR319 was reported to be downregulated in *G. hirsutum* and *A. mongolicus* (Xie et al., 2015; Gao et al., 2016). Transgenic *Agrostis stolonifera*

overexpressing miR319 showed morphological changes and exhibited enhanced drought tolerance associated with increased leaf wax content and water retention but reduced sodium uptake (Zhou et al., 2013). Elevated levels of miR319 were associated with enhancing the capacity of *O. sativa* to withstand water stress (Zhou et al., 2010). Similar kind of deductions can be drawn for miR319 accumulation in the current study. miR159 showed reduction in *in situ* and *ex situ* stressed *I. campanulata*, and *in situ* stressed *J. pentantha*. Previously it was reported to be downregulated in *O. sativa* and *Solanum tuberosum*, whereas upregulated in *A. thaliana*, and *P. trichocarpa* (Zhou et al., 2010; Yang et al., 2014; Reyes and Chua, 2007; Shuai et al., 2013). Reduction in levels of miR159 and associated upregulation of its target GAMYB like genes were considered to have role in response to water stress (Yang et al., 2014). miR858 even targets MYB transcription factors and was only found to be upregulated in drought stressed *I. campanulata*. In *A. thaliana*, miR858 negatively regulates its target AtMYB20, which is found to be suppressed by drought stress. MYB20 loss of function mutant *A. thaliana* plants displayed resistance to drought (Shuai et al., 2013). Therefore accumulation of miR858 in *I. campanulata* can probably have species specific drought tolerance function.

Drought responsive miR169 is one of the largest miRNA families that is conserved in all plant species (Zhang, 2015). In present study, although the expression pattern of miR169 was not clear by sequencing, its expression by blot analysis was very clear. It was downregulated under both *in situ* and *ex situ* water deficit in *I. campanulata*. However this was not the case in *J. pentantha*, as miR169 levels were reduced under *in situ* stress (inconspicuous than *I. campanulata*) and upregulated under *ex situ* stress. Dehydration prompted miR169 downregulation was noticed in *A. thaliana*, *P. persica*, *P. virgatum* and *M. truncatula* (Li et al., 2008; Eldem et al., 2012; Sun et al., 2012; Trindade et al., 2010). miR169 regulates the expression of Nuclear Factor Y (NF-Y) subunit A 5 (NFYA5) transcription factors. Reduction in miR169 and corresponding over expression of NFYA5 was found to increase drought tolerance (Li et al., 2008). Likewise, downregulation of miR169 may be associated with better drought tolerance of *I. campanulata*. Contrarily, upregulation of miR169 was reported in *O. sativa*, *G. max*, *P. euphratica* and *Solanum lycopersicum* (Li et al., 2011; Qin et al., 2011; Zhang et al., 2011; Zhou et al., 2010). NFYA5 knockout plants and those overexpressing miR169 showed enhanced leaf water loss, making it drought sensitive (Li et al.,

2008). Similarly, response of miR169 in *ex situ* stressed *J. pentantha* may be linked with its drought sensitivity.

miR408 and miR398 are highly conserved, abiotic stress responsive miRNAs that regulate genes encoding copper proteins (Abdel-Ghany and Pilon, 2008; Trindade et al., 2010). miR408 was upregulated in *ex situ* water stress *I. campanulata* while downregulated in *J. pentantha*. Previously miR408 was reported to accumulate in drought stressed *A. thaliana*, *M. truncatula*, *H. vulgare* and *C. arietinum* (Liu et al., 2008; Trindade et al., 2010; Kantar et al., 2010; Hajyzadeh et al., 2014). Amongst the *O. sativa* genotypes under drought stress, miR408 levels were only elevated in drought tolerant ones (Nagina-22 and Vandana) but not in drought-sensitive genotypes (Mutum et al., 2013). Corresponding to the levels of miR408 in *O. sativa*, the target plantacyanin-like protein showed inverse expression profile (Mutum et al., 2013). In response to drought stress, *C. arietinum* overexpressing miR408 showed normal growth whereas the control once showed severe symptoms of stress (Hajyzadeh et al., 2014). It clearly indicates that induction of miR408 is important for drought tolerance, which was even portrayed by the drought tolerant species *I. camapanulata*. Strangely under *in situ* stress, miR408 showed major downregulation in *I. campanulata*. Such differential regulation of miR408 can be attributed for its responsiveness towards various environmental stresses such as salinity, heat, oxidative stress, drought and osmotic stress; which under *in situ* conditions can possibly influence the expression of miR408 (Mutum et al., 2013; Ma et al., 2015). Similar to miR408, regulation of miR398 is orchestrated by various abiotic stressors like heat, salinity, cold, oxidative stress, cadmium stress and drought (Trindade et al., 2010; Frazier et al., 2011; Kantar et al., 2011; Zhou et al., 2012; Guan et al., 2013). miR398 is known to target CSD, COX5b and CCS (Sunkar et al., 2006; Sunkar and Zhu, 2004; Chu et al., 2005). miR398 in *I. camapanulata* was upregulated under *ex situ* stress whereas downregulated under *in situ* stress. *Vice versa* regulation was seen in *J. pentantha*. Differential response of miR398 was even observed in drought stressed *M. truncatula*, wherein Trindade et al., (2010) observed upregulation of miR398 while Wang et al., (2011) showed downregulation. Such contrasting observations in the same plant species can be explained by the extent and duration of drought stress. Similar explanation can be considered for the differential response of miR398 in the two Convolvulaceae species.

Overall, the miRNA expression study showed that although most of the miRNA families were conserved between the two Convolvulaceae species, their basal expression was very different. Both *in situ* and *ex situ* drought triggered differential regulation of miRNAs in the drought tolerant-wild and sensitive-cultivated species. Drought responsiveness of several highly conserved miRNAs was similar under *in situ* and *ex situ* stress in the tolerant- *I. campanulata*, which was not the case with sensitive-*J. pentantha*. Similar to *J. pentantha* differential expression of miRNAs was observed in sugarcane that is grown in greenhouse *vs.* field conditions (Gentile et al., 2015). Regulation of miR172, miR160, miR167, miR393, miR159, miR858 and miR169 can be considered as the major drivers of drought tolerance in *I. campanulata*.