# CHAPTER-III

# RESULTS

### 3.1. Soil water status at control and experimental site (*in situ* conditions)

Soil water content influences plant growth, development and productivity. Average soil water content was observed to vary across the soil depths (i.e. surface, 20-30cm and 30-40cm) at both the field sites. Soil water content at both the sites was very low at the surface layer (Figure 5). At subsequent depth (i.e. 20-30cm) the soil water content was moderately higher than surface; followed by prominently higher water content at 30-40 cm depth (Figure 5). Apart from variations across soil profiles, soil water content also varied in between the two sites. Amongst the two sites, the average soil water content at experimental site was 60%, 51.6%, 47.8% lesser at surface, 20-30cm and 30-40cm soil depths respectively than observed at control site (Figure 5). Results indicate low soil water content at experimental site (for all the analyzed soil depths) than compared to control.

### 3.2. Vegetation characteristics at control and experimental site (*in situ* conditions)

General vegetation characteristics have been studied to ensure that *in situ* variability in soil water content is a generic phenomenon and not a one off kind, seen only in seasonal herbaceous cover. Composition of vegetation at the two field sites was nearly similar. Species richness of trees found growing at the two sites was almost similar; however the density was comparatively higher at control site. This may be considered one of the reasons in maintaining relatively high levels of soil water content at control site. Shrubs and herbs dominated the two sites. Some of these plant species were cultivated, largely coming from agricultural activities, while others were wild. *Withania somnifera*, *Caesalpinia pulcherrima, Hibiscus rosa-sinensis, Tecoma stans*, *Adhatoda vasica and Cassia alata* were cultivated-shrub species commonly found growing at both the field sites. Wild-shrub species largely observed growing at the two field sites included *Accasia nilotica, Datura stramonium, Calotropis procera, Calotropis gigantea* and *Ipomoea carnea*. Five random quadrats of 5x5m were used for sampling the shrub species at the two field sites. Species richness, density, height, stem diameter (at ground level), canopy spread and leaf/leaflet area of these species were measured (Figure 6). Quadrat sampling was carried out during vegetative growth phase of most of the species. Species richness of shrubs observed at control and experimental site was almost similar (Table 1a, Table 1b). However density and the

range of values for plant height, stem diameter, and leaf/leaflet area of these species were different between the two sites. The density of shrubs at experimental site was lesser than compared to control site (Table 1a, Table 1b). Average height and stem diameter of the shrubs was observed to be lower at experimental site than compared to control (Table 1a, Table 1b), signifying the difference in the growth and vigor of these species at the two sites. Shrubs growing at experimental site were observed to show smaller leaf/leaflet area than compared to the ones growing at control site (Table 1a, Table 1b). Results indicate differences in the vegetative characteristics of shrubs growing at the two field sites.

Most of the herbaceous species growing at the two field sites were wild, which includes *Corchorus capsularis*, *Corchorus acutangulus*, *Ruellia nudiflora*, *Euphorbia hirta*, *Sida acutifolia*, *Acylypha indica, Tephrosia purpuria*, *Parthenium hysterophorus*, *Cassia tora*, *Indigofera cordifolia*, *Achyranthes aspera*, *Commelina nudiflora*, *Ipomoea campanulata, Ipomoea pes-tigridis*, *Ipomoea aquatic*, *Cynodon dactylon, Xanthium strumarium* and *Tridax procumbens*. Cultivated-herb species found growing at the two sites included *Vinca rosea*, *Jacquemontia pentantha*, *Clitoria ternatea, Antigonon leptopus*, *Gossipium herbaceum*, *Quisqualis indica*, *Helianthus annuus* and *Tylophora indica*. For sampling, 1x1m quadrats were laid to record species richness, density, height, stem diameter (at ground level) and leaf/leaflet area at both the sites (Figure 6). Species richness of herbaceous vegetation at control and experimental site was observed to be almost similar (Table 2a, Table 2b), indicating resemblance in the herb species growing at the two sites. However, lower density of these herb species at experimental site has been observed than compared to control. The range of values for height, stem diameter and leaf/leaflet area for these species growing at experimental site was also observed to be different than control site. Average height and stem diameter of herbs was observed to be lower at experimental site as compared to control (Table 2a, Table 2b), indicating difference in the growth and vigor of these species growing at the two sites. Leaf/leaflet area of these species growing at the experimental site was observed to be profoundly lower than compared to control (Table 2a, Table 2b). Based on these observations, few wild and cultivated species were selected for an elaborate study.

Figure 5 Soil water content measured at the two field sites (at different depths). Data are ±SD (n=10).

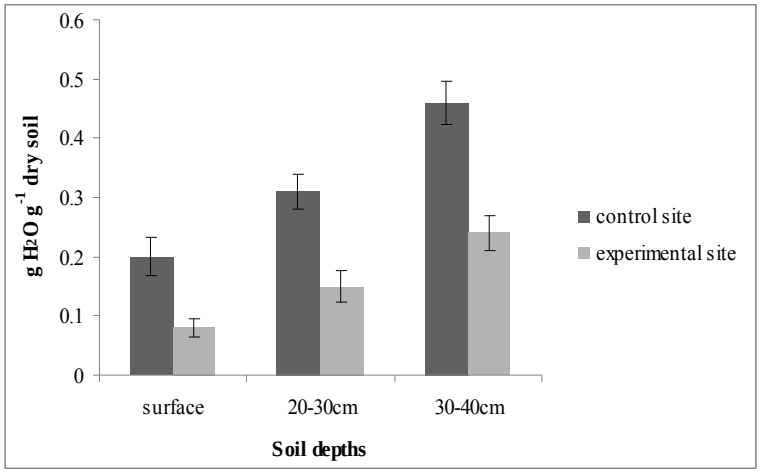



Figure 6 Quadrat sampling for measuring species richness, density, height, stem diameter and leaf/leaflet area.

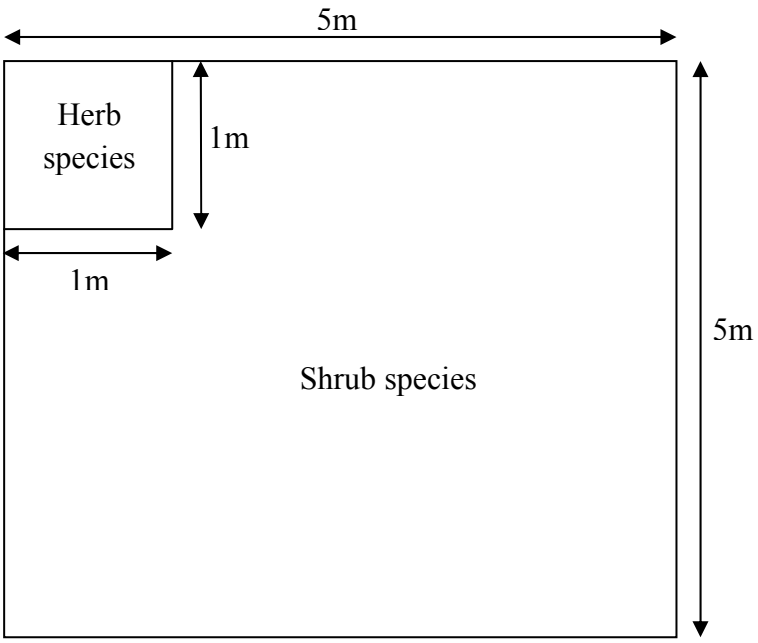


Table 1 Vegetative characteristics of shrub species growing at (a) control site and (b) experimental site

(a)

| Parameters | Quadrat 1 | Quadrat 2 | Quadrat 3 | Quadrat 4 | Quadrat 5 |
|---|---|---|---|---|---|
| Species richness | 3 | 2 | 2 | 1 | 3 |
| Density | 4 | 5 | 6 | 5 | 4 |
| Height (range) (m) | 2-5 | 2.1-5.8 | 2.5-6.3 | 2-5.5 | 2.6-7 |
| Stem diameter at ground level (range) (cm) | 7-30 | 7.2-31 | 7-31 | 8-30 | 7-30 |
| Leaf/leaflet area (range) ($cm^2$) | 2.9-110 | 3.5-126 | 2.8-120 | 3.2-110 | 2.7-118 |

(b)

| Parameters | Quadrat 1 | Quadrat 2 | Quadrat 3 | Quadrat 4 | Quadrat 5 |
|---|---|---|---|---|---|
| Species richness | 2 | 3 | 3 | 2 | 1 |
| Density | 3 | 2 | 4 | 1 | 2 |
| Height (range) (m) | 1.8-4.9 | 1.5-5 | 2-4.8 | 1.7-5.2 | 1.8-4.5 |
| Stem diameter at ground level (range) (cm) | 6.8-27 | 7-29 | 6.1-28 | 6-27.3 | 5.8-27 |
| Leaf/leaflet area (range) ($cm^2$) | 2.7-85 | 2.3-94 | 3-82 | 2.1-90 | 1.5-96 |

Table 2 Vegetative characteristics of herb species growing at (a) control site and (b) experimental site

(a)

| Parameters | Quadrat 1 | Quadrat 2 | Quadrat 3 | Quadrat 4 | Quadrat 5 |
|---|---|---|---|---|---|
| Species richness | 6 | 8 | 8 | 10 | 6 |
| Density | 21 | 18 | 15 | 23 | 21 |
| Height (range) (m) | 0.4-2 | 0.5-2.3 | 0.4-2.5 | 0.5-2.7 | 0.4-2.1 |
| Stem diameter at ground level (range) (cm) | 0.8-3.2 | 0.9-2.5 | 1-2.1 | 0.7-2.9 | 1-2.9 |
| Leaf/leaflet area (range) ($cm^2$) | 2.3-92.1 | 2.2-85.3 | 2.4-97.1 | 2.3-95.3 | 2.5-85 |

(b)

| Parameters | Quadrat 1 (1x1m) | Quadrat 2 (1x1m) | Quadrat 3 (1x1m) | Quadrat 4 (1x1m) | Quadrat 5 (1x1m) |
|---|---|---|---|---|---|
| Species richness | 6 | 8 | 7 | 6 | 9 |
| Density | 17 | 14 | 18 | 12 | 14 |
| Height (range) (m) | 0.2-2 | 0.2-1.8 | 0.3-1.7 | 0.3-2 | 0.4-1.7 |
| Stem diameter at ground level (range) (cm) | 0.7-2 | 0.8-1.7 | 0.7-2.4 | 0.6-2.5 | 0.6-2.7 |
| Leaf/Leaflet area (range) $cm^2$) | 1.8-84.2 | 1.8-82.1 | 1.7-73 | 2.1-81 | 1.9-84.1 |

### 3.2.1. Phenotypic variations in few selected plant species growing at control and experimental site (*in situ* conditions)

Variations in phenotypic traits were observed in the six wild and six cultivated plant species (each belonging to Solanaceae, Fabaceae, Asteraceae, Malvaceae, Apocynaceae, Convolvulaceae) belonging to similar growth phase. Leaf/leaflet area and perimeter was smaller in selected wild and cultivated plant species at experimental site than compared to control. Wild species at experimental site showed almost 13-51% lesser leaf area than control (Table 3a, Table 3b). While cultivated species at experimental site showed 20-68% lesser leaf area as compared to control (Table 3c, Table 3d) which indicated relatively higher reduction in these plants. The reduction in leaf perimeter was observed to be relatively prominent amongst the cultivated species (i.e. almost 29-44%) than the wild species (i.e. almost 21-35%) growing under water deficit (Table 3a, Table 3b, Table 3c, Table 3d). Similarly, leaf/leaflet length and width were also observed to be different in the selected wild and cultivated species growing under water deficit. Maximum range of decrease in leaf width (i.e. 25-49%) was observed in cultivated species than in wild species (i.e. 18-38%) exposed to water deficit prevailing at experimental site (Table 3a, Table 3b, Table 3c, Table 3d). The percentile decrease in leaf length under water deficit was almost similar in wild (23-44%) and cultivated (28-44%) species (Table 3a, Table 3b, Table 3c, Table 3d). SLA (Specific leaf area), an important indicator of plant resource use strategy, was observed to be different in wild and cultivated species growing at experimental and control site. SLA was observed to decrease in cultivated species by 31-59% and 17-48% in wild species (Table 3a, Table 3b, Table 3c, Table 3d). It indicated conspicuous decrease in SLA under water deficit in cultivated species. In contrast to SLA, LDMC (Leaf dry matter content) was observed to increase in all the selected species under the influence of water deficit. Water stressed wild species showed 24-52% increase in LDMC while 21-54% increase was seen in cultivated species (Table 3a, Table 3b, Table 3c, Table 3d). It suggested almost similar increase in LDMC in both the groups.

### 3.2.2. Phenotypic variations in *I. campanulata* and *J. pentantha* growing at control and experimental site (*in situ* conditions)

Amongst the selected wild and cultivated species under study, *I. campanulata* (wild) and *J. pentantha* (cultivated) belonging to family Convolvulaceae showed distinct variation in the measured phenotypic traits in response to water deficit. As compared to *J. pentantha*, *I. campanulata* showed inconspicuous reduction in leaf area (13.2%) and leaf perimeter (21.4%) under water stress as compared to control (Table 3a, Table 3b, Table 3c, Table 3d). The leaf length and leaf width showed 23.1% and 18.3% reduction in water stressed *I. campanulata* respectively, while it was 34.3% and 32.1% in water stressed *J. pentantha* respectively (Table 3a, Table 3b, Table 3c, Table 3d). It suggested that both the species showed relatively prominent reduction in leaf length than width under water deficit. SLA even showed reduction in both the species under stress; however it was predominantly higher in *J. pentantha* (59.3%) than *I. campanulata* (47.8%) (Table 3a, Table 3b, Table 3c, Table 3d). Contrary to reduction in these leaf parameters, LDMC was observed to increase by 33.2% and 29.2% in water stressed *I. campanulata* and *J. pentantha* respectively (Table 3a, Table 3b, Table 3c, Table 3d). It portrayed relatively higher increase in LDMC in wild species under stress.

Apart from phenotypic variations in measured leaf traits, *I. campanulata* and *J. pentantha* also showed distinct variations in reproductive traits under water deficit. *I. campanulata* and *J. pentantha* growing under water deficit showed almost similar (20%) reduction in length of corolla as compared to the control site (Table 4). However the reduction in length of pistil and stamen was different in the two species. *I. campanulata* showed 6.7% and 6.5% reduction in length of pistil and stamen respectively (Table 4). *J. pentantha* showed 35% and 7.1% reduction in length of pistil and stamen respectively (Table 4).

Table 3 Phenotypic variations (leaf traits) observed in selected wild and cultivated species growing at control and experimental site. Tables (a) and (b) represents variations in wild species growing at control and experimental site respectively; and Tables (c) and (d) represents variations in cultivated species growing at control and experimental site respectively. Data are ±SD (n=10).

(a)

| Wild Species | Control site | | | | | |
|---|---|---|---|---|---|---|
| | perimeter (cm) | area ($cm^2$) | length (cm) | width (cm) | SLA ($cm^2g^{-1}$) | LDMC |
| *D. stramonium* | 30.5±3.4 | 69.6±4.4 | 12.2±2.9 | 8.7±1.5 | 248.4±6.1 | 0.17±0.02 |
| *T. purpuria* | 6.4±0.5 | 2.3±0.3 | 2.1±0.3 | 1.4±0.08 | 202.1±2.1 | 0.37±0.03 |
| *P. hysterophorus* | 29.2±2.9 | 17.4±1.7 | 9.3±0.8 | 4.1±0.4 | 223.9±5.3 | 0.20±0.01 |
| *S. acutifolia* | 11.6±0.8 | 7.6±0.9 | 4.2±0.4 | 2.5±0.1 | 203.3±4.7 | 0.32±0.04 |
| *C. procera* | 29.9±2.5 | 73.5±5.2 | 12.1±0.7 | 8.1±0.5 | 182.1±4.2 | 0.18±0.02 |
| *I. campanulata* | 33.6±4.1 | 98.1±4.0 | 15.0±2.1 | 9.6±0.6 | 274.9±4.0 | 0.19±0.01 |

(b)

| Wild Species | Experimental site | | | | | |
|---|---|---|---|---|---|---|
| | perimeter (cm) | area ($cm^2$) | length (cm) | width (cm) | SLA ($cm^2g^{-1}$) | LDMC |
| *D. stramonium* | 20.3±3.8 | 37.7±2.6 | 7.8±2.7 | 6.1±0.5 | 172.3±4.5 | 0.24±0.01 |
| *T. purpuria* | 4.3±0.7 | 1.3±0.5 | 1.4±0.05 | 1.1±0.5 | 143.7±2.2 | 0.49±0.01 |
| *P. hysterophorus* | 19.9±2.2 | 8.9±1.1 | 6.8±0.8 | 2.8±0.2 | 132.8±6.2 | 0.32±0.006 |
| *S. acutifolia* | 7.4±0.5 | 3.7±1.0 | 2.3±0.7 | 1.5±0.3 | 168.6±3.2 | 0.45±0.02 |
| *C. procera* | 19.5±3.1 | 48.7±2.0 | 7.3±0.6 | 5.3±0.8 | 135.3±6.0 | 0.38±0.003 |
| *I. campanulata* | 26.3±2.2 | 85.0±2.6 | 11.5±0.7 | 7.8±0.6 | 143.3±3.5 | 0.28±0.02 |

(c)

| Cultivated species | Control Site | | | | | |
|---|---|---|---|---|---|---|
| | perimeter (cm) | area ($cm^2$) | length (cm) | width (cm) | SLA ($cm^2g^{-1}$) | LDMC |
| *W. somnifera* | 51.8±4.2 | 139.8±6.3 | 22.9±2.0 | 8.9±1.2 | 394.9±5.1 | 0.11±0.02 |
| *C. ternatea* | 8.4±1.2 | 5.2±1.2 | 4.1±0.6 | 2.4±0.2 | 345.7±2.7 | 0.26±0.008 |
| *H. annuus* | 9.2±0.8 | 4.9±0.8 | 2.9±0.5 | 2.6±0.2 | 102.7±1.2 | 0.18±0.003 |
| *G. herbaceum* | 29.2±2.4 | 82.1±5.2 | 10.7±1.3 | 11.3±1.2 | 277.8±5.2 | 0.20±0.007 |
| *T. indica* | 13.1±2.9 | 18.2±1.5 | 5.4±0.5 | 4.2±0.1 | 238.9±3.4 | 0.15±0.01 |
| *J. pentantha* | 13.2±2.2 | 24.1±3.2 | 6.7±0.7 | 5.3±0.5 | 283.2±2.6 | 0.18±0.02 |

(d)

| Cultivated species | Experimental Site | | | | | |
|---|---|---|---|---|---|---|
| | perimeter (cm) | area ($cm^2$) | length (cm) | width (cm) | SLA ($cm^2g^{-1}$) | LDMC |
| *W. somnifera* | 28.8±3.6 | 45.06±8.3 | 14.7±3.2 | 4.5±0.5 | 259.9±6.2 | 0.24±0.01 |
| *C. ternatea* | 5.2±0.8 | 2.80±0.8 | 2.3±0.3 | 1.8±0.2 | 233.1±3.2 | 0.33±0.02 |
| *H. annuus* | 5.6±1.0 | 1.91±0.5 | 1.8±0.4 | 1.7±0.2 | 56.3±1.0 | 0.24±0.007 |
| *G. herbaceum* | 20.6±1.6 | 43.9±3.6 | 7.7±1.0 | 8.5±0.7 | 157.2±3.8 | 0.28±0.01 |
| *T. indica* | 7.8±2.2 | 10.5±2.6 | 3.7±1.3 | 2.6±0.4 | 163.5±4.5 | 0.21±0.02 |
| *J. pentantha* | 8.7±1.0 | 19.1±1.7 | 4.4±0.2 | 3.6±0.3 | 115.2±4.8 | 0.26±0.02 |

Table 4 Phenotypic variations (floral traits) observed in *I. campanulata* and *J. pentantha* growing at control and experimental site. Data are ±SD (n=10)

| Plant species | Control site | | | Experimental site | | |
|---|---|---|---|---|---|---|
| | Corolla length (cm) | Pistil length (cm) | Stamen length (cm) | Corolla length (cm) | Pistil length (cm) | Stamen length (cm) |
| *I. campanulata* | 9.6± 0.2 | 3± 0.1 | 3.1± 0.2 | 7.5± 0.2 | 2.8± 0.2 | 2.9± 0.4 |
| *J. pentantha* | 2± 0.1 | 2± 0.2 | 1.4± 0.2 | 1.6± 0.2 | 1.3± 0.1 | 1.3± 0.7 |

## 3.3. Physiological and biochemical response of *I. campanulata* and *J. pentantha* (*in situ* conditions)

Physiological and biochemical responses of *I. campanulata* and *J. pentantha* exposed to *in situ* water stress, were examined by analyzing the alterations in selected parameters such as chlorophyll and anthocyanin contents, levels of lipid peroxidation and SOD isoforms.

### 3.3.1. Chlorophyll content

Levels of chlorophyll were observed to be different in the two selected species at control and experimental site. Under the influence of water deficit, both the species showed low levels of chlorophyll content than compared to control. The level of chlorophyll content in *I. campanulata* growing at experimental site was 18.6% lesser than observed at control site (Table 5). While, *J. pentantha* growing at experimental site showed 53.4% lesser levels of chlorophyll as compared to control (Table 5).

### 3.3.2. Anthocyanin content

Concentration of anthocyanin symbolizes the stress experienced by a plant species. In the current study, levels of anthocyanin were observed to increase in both the species growing at experimental site as compared to control. However the degree of anthocyanin accumulation in response to water deficit varied between the two species. The concentration of anthocyanin was observed to be elevated by 98.1% in *I. campanulata* growing at experimental site than compared to control (Table 5). *J. pentantha* growing at experimental site showed 195.4% accumulation of anthocyanin than control (Table 5). Results indicate relatively high levels of anthocyanin accumulation in *J. pentantha* than *I. campanulata* in response to water stress prevailing at experimental site.

### 3.3.3. Lipid peroxidation

Lipid peroxidation (measuring the oxidative levels) was estimated in the two species growing at control and experimental site. Levels of lipid peroxidation were measured in terms of MDA concentration. Both the species exposed to water deficit at experimental site showed high levels of MDA than measured at control site. However, these levels of rise in MDA concentration varied between the two species. *I.*

*campanulata* exposed to water deficit showed 22.2% higher levels of MDA than compared to control (Table 5). The levels of MDA were observed to be 60% higher in *J. pentantha* growing at experimental site than control (Table 5). Results indicate predominant rise in levels of MDA in *J. pentantha* than compared to *I. campanulata* under similar conditions of water stress.

Table 5 Chlorophyll, anthocyanin and MDA concentration in *I. campanulata* and *J. pentantha* growing at control and experimental site. Data are ±SD (n=3).

| Parameters | Control site | | Experimental site | |
|---|---|---|---|---|
| | *I. campanulata* | *J. pentantha* | *I. campanulata* | *J. pentantha* |
| Chlorophyll mg $g^{-1}$ fresh weight | 15.2±0.71 | 6.57±0.32 | 12.37±0.47 | 3.06±0.44 |
| Anthocyanin ug $g^{-1}$ fresh weight | 0.53±0.08 | 1.31±0.06 | 1.05±0.06 | 3.87±0.04 |
| MDA n mol $g^{-1}$ fresh weight | 14.52±1.54 | 12.92±2.21 | 17.74±1.55 | 20.67±1.15 |

### 3.3.4. Superoxide dismutases

SOD levels were estimated in both the species growing under control and water deficit. Based on the metal cofactor involved in the formation of the enzyme, they are classified as MnSOD, FeSOD and CuZnSOD. Using activity staining of PAGE, a total of 8 SOD isoforms (2 MnSODs, 2 FeSODs and 4 CuZnSODs) were identified in both the species (Figure 7a). Band intensities of these SOD isoforms were analyzed using the AlphaEaseFC software. Relative band intensity of all SOD isoforms (i.e. band intensity of water deficit samples to that of control) were calculated for both the species. Under water deficit, MnSOD isoforms showed a transient but significantly higher activity in both the species ($P<0.05$) (Figure 7a, Figure 7b). Activity of MnSODI and MnSODII was 17.6% and 22.4% higher in *I. campanulata* respectively (Figure 7b). *J. pentantha* under water deficit showed 12.7% and 18.3% higher activity of MnSODI and MnSODII respectively than control plants (Figure 7b). Both FeSODI and FeSODII isoforms showed significant rise in their activity in *I. campanulata* under the influence of water stress ($P<0.05$) (Figure 7a, Figure 7c). The activity of FeSODI and FeSODII was 19.6% and 33.2% higher in *I. campanulata* respectively (Figure 7c). However, water stressed *J. pentantha* showed significant rise in the activity of only FeSODII ($P<0.05$), while rise in the activity of FeSODI was

insignificant (P>0.05) (Figure 7c). Rise in the activity of FeSODII was observed to be 23% in *J. pentantha* exposed to water deficit (Figure 7c). CuZnSOD isoforms showed significant induction in their activity in both the species exposed to water deficit as compared to control (P<0.05) (Figure 7a, Figure 7d). The activity of CuZnSODI, CuZnSODII, CuZnSODIII, and CuZnSODIV was observed to be induced by 45%, 60%, 52.5% and 36.4% respectively in water stressed *I. campanulata* (Figure 7d). While the rise in the activity of CuZnSODI, CuZnSODII, CuZnSODIII, and CuZnSODIV was 16.3%, 14%, 34.5% and 10.7% respectively in water stressed *J. pentantha* (Figure 7d). It indicated prominent induction in the activities of CuZnSOD isoforms in both the species growing under water deficit. However the rise in the activity of these CuZnSOD isoforms was prominently higher in *I. campanulata* than compared to *J. pentantha*.

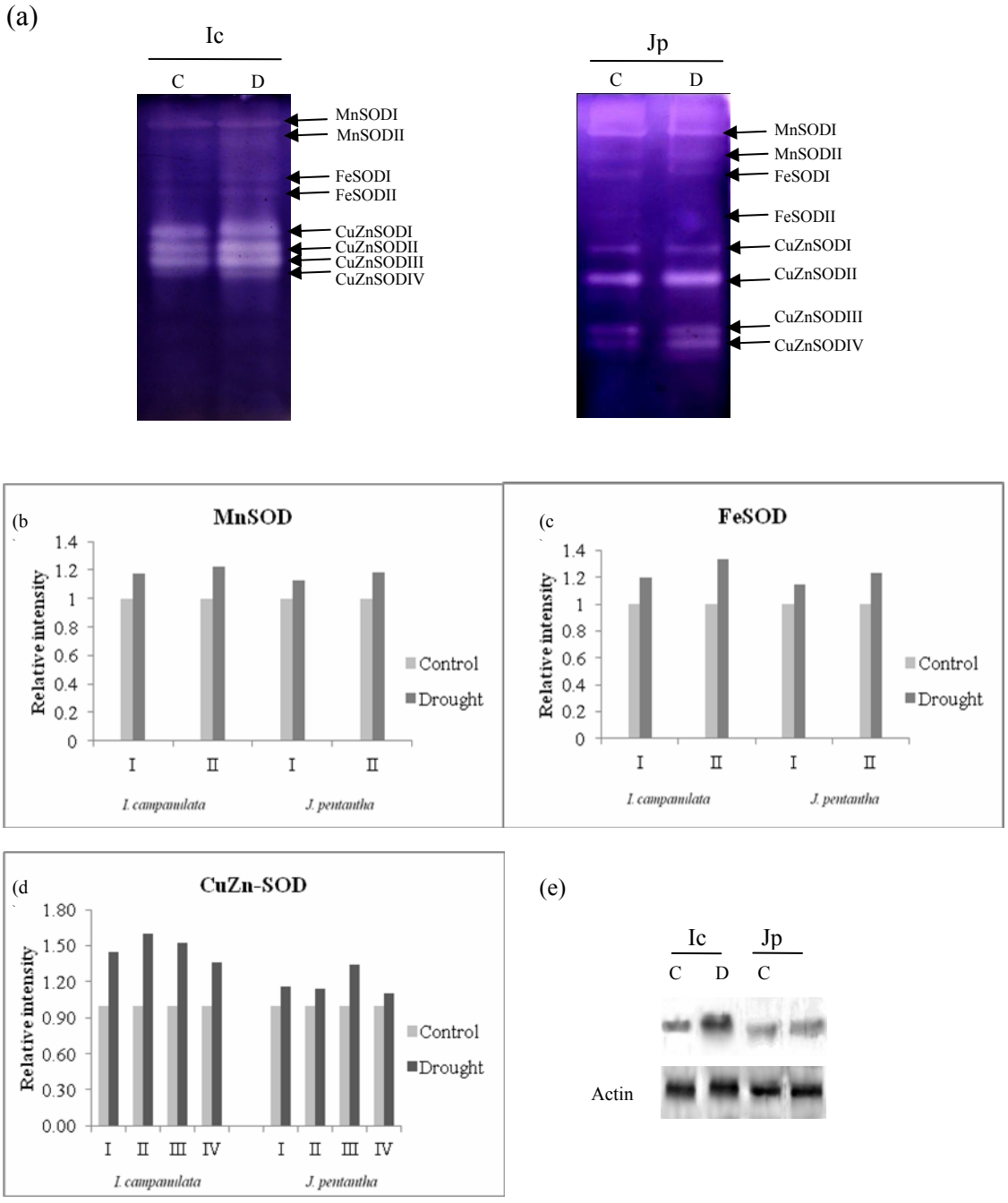
### 3.3.5. Detection of CuZnSOD through immunoblot (Western blotting)

Immunoblot analysis was performed using polyclonal anti-CSD2 antiserum. Relative band intensities of CuZnSODs showed a prominent induction of CuZnSOD in *I. campanulata* as compared to *J. pentantha* under water deficit (Figure 7e). These immunoblot results confirmed the differential rise in the activity of CuZnSOD isoforms in both the species as observed in-gel activity assay.

## 3.4. Statistical analysis

The statistical test demonstrated whether the difference between measured values of soil water content, chlorophyll content, anthocyanin concentration, MDA concentration and relative band intensities of SOD isoforms at control and experimental site were significantly different or not. One way analysis of variance (ANOVA) performed showed that the difference between the values (for above mentioned parameters) of experimental and control site were significant (P<0.05) or insignificant (P>0.05). The test was extended to evaluate whether the variations seen in the two selected species are significantly different or not (at 0.05 level).

Figure 7 SOD isoforms of *I. campanulata* (Ic) and *J. pentantha* (Jp) growing under control (C) and *in situ* water deficit conditions (D) as anlysed by (a) activity staining of native PAGE. Relative intensity of (b) MnSOD, (c) FeSOD and (d) CuZnSOD isoforms in *I. campanulata* and *J. pentantha* exposed to water deficit than compared to control. (e) Immunoblot showing level of CuZnSOD in *I. campanulata* and *J. pentantha* growing under water deficit. Actin was used as loading control.



### 3.5. Quality of total RNA extracted from *I. campanulata* and *J. pentantha* growing under *in situ* conditions

Total RNA extracted from leaf tissue of both the species growing at control and experimental site were resolved on 1% agarose gel. Results showed three distinct bands of 5S, 18S and 28S rRNA. The intensity of 28S rRNA band was observed to be almost double to that of 18S rRNA bands indicating RNA extracted was almost pure. Further the total RNA quality was analysed through NanoDrop™ 1000 Spectrophotometer. The ratio $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ showed a value ranging between 1.7-1.9. These results indicated good (but not excellent) quality of total RNA extracted from both the species. The quality of total RNA may be affected by the high levels of secondary metabolites found in plants growing under *in situ* conditions.

#### 3.5.1. Sequencing of Small RNA libraries from *I. campanulata* and *J. pentantha* growing under *in situ* conditions

Four small RNA libraries were generated from the total RNA of foliar samples of *I. campanulata* and *J. pentantha*, growing at the two field sites. They were sequenced using Illumina-high throughput sequencing technology at LC Sciences. However, these samples could not be processed to identify the sequences. It was due to lower purity of total RNA as compared to the desirable quality.

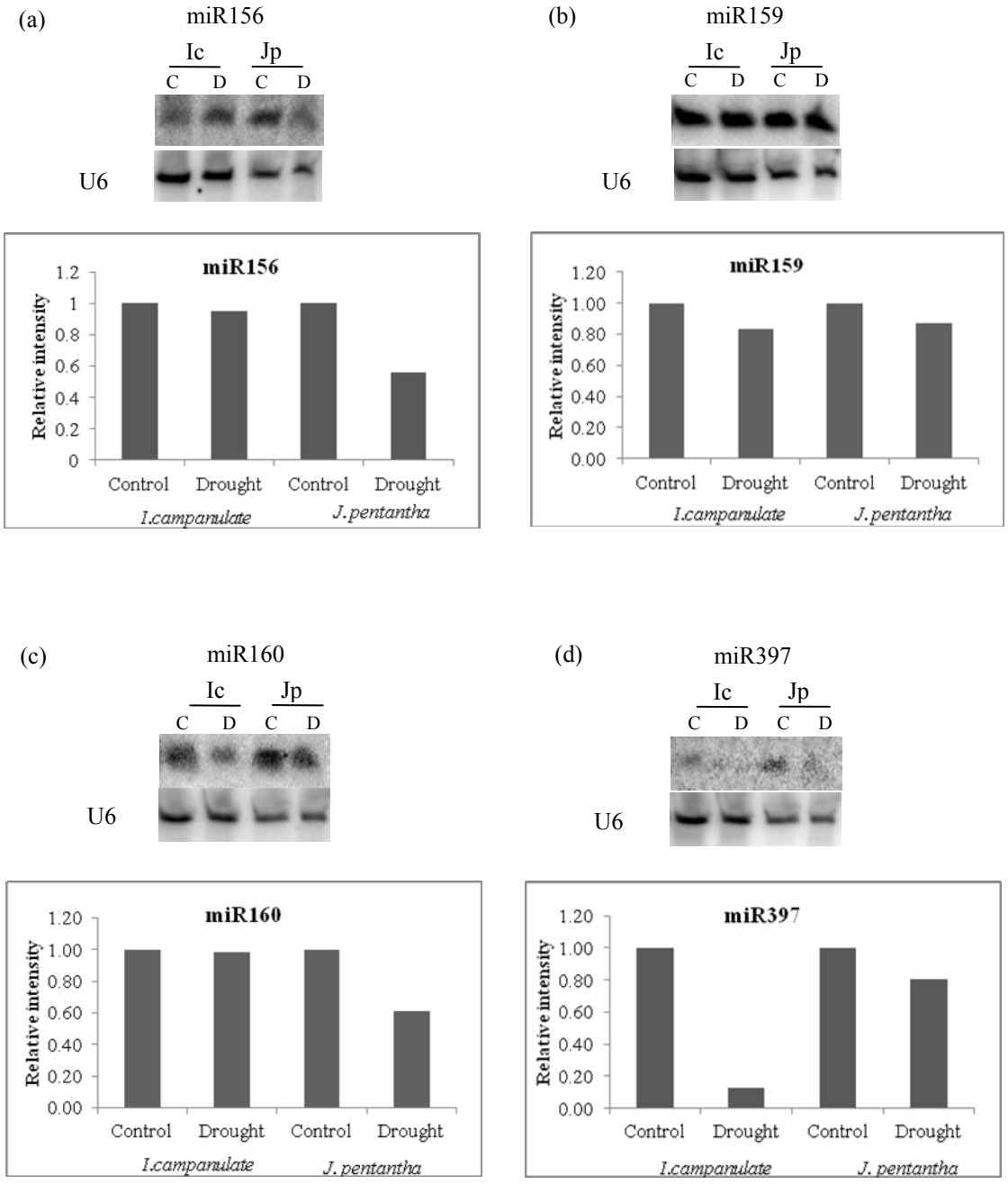
#### 3.5.2. miRNA blot analysis of *I. campanulata* and *J. pentantha* (*in situ* conditions)

Although small RNAs could not be sequenced from these samples, Northern blotting was carried out in order to analyze the expression pattern of few of the highly conserved miRNAs in both the species in response to water deficit. The blots were probed for conserved miRNA sequences of *Arabidopsis* which were end labeled with $\gamma$-$^{32}$P-ATP. The images acquired by scanning the phosphor screen with Typhoon scanner were loaded into the software and band intensities were obtained. Each of the band intensities were normalized in accordance to U6 (small nuclear RNA) that was used as loading control. The relative band intensities (i.e. band intensity of water deficit samples to that of control) for each conserved miRNA of both the species were analyzed and calculated. Results revealed that miR156, miR159, miR160, miR397, miR168, miR171, miR319, miR398, miR408, miR169, miR172, miR396, miR393 and miR167 were downregulated; and miR395 was upregulated in response to water

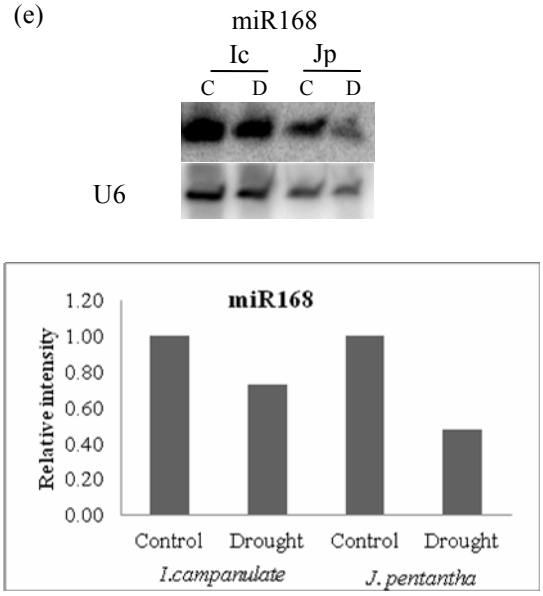
deficit in *I campanulata* (Figure 8). Results portrayed that majority of highly conserved miRNAs (under study) showed reduction in their expression in reaction to water stress in *I. campanulata*. miR408 showed major downregulation, wherein it was downregulated by 7.55 fold (Figure 8i). Followed by miR408, miR397 showed 6.70 fold downregulation in response to stress (Figure 8d). Other than these two miRNAs, miR167, miR396 and miR171 also showed reduced levels in the range of 2-4 fold under water deficit (Figure 8o, Figure 8l, Figure 8f). Reduction in levels of miR172, miR398 and miR169 ranged in between 1-1.5 fold (Figure 8k, Figure 8h, Figure 8j). While miR159, miR168 and miR319 showed reduction in range of 0.2-0.38 fold (Figure 8b, Figure 8e, Figure 8g). Lastly miR393, miR160 and miR156 showed marginal reduction in their levels (0.02-0.05 fold) under water stress (Figure 8m, Figure 8c, Figure 8a). Contrary to this, miR395 showed upregulation (0.65 fold) in response to stress (Figure 8n).

*J. pentantha*, subjected to water deficit conditions, showed reduction in levels of miR156, miR159, miR160, miR397, miR168, miR171, miR169, miR396, miR393, miR395 and miR167; while miR319, miR398, miR408 and miR172 showed increase in their levels (Figure 8). Results revealed that majority of conserved miRNAs (observed here) showed reduction in their expression under water deficit conditions. Amongst these miRNAs, miR171 showed major downregulation (6.69 fold) in response to water stress in *J. pentantha* (Figure 8f). miR167 and miR393 showed reduction in their expression by 2.78 and 1.83 fold respectively under stressful conditions (Figure 8o, Figure 8m). miR168 and miR396 showed almost similar (1.08 fold) levels of reduction under stress (Figure 8e, Figure 8l). Several other drought responsive miRNAs including miR156, miR160, miR397, miR159, miR169 and miR395 showed reduction in their levels by 0.78, 0.64, 0.25, 0.15, 0.33 and 0.07 fold respectively (Figure 8a, Figure 8c, Figure 8d, Figure 8b, Figure 8j, Figure 8n). Opposite to these observations, miR398, miR408, miR172 and miR319 showed increase in their expression by 1.3, 0.73, 0.36 and 0.21 fold respectively (Figure 8h, Figure 8i, Figure 8k, Figure 8g).

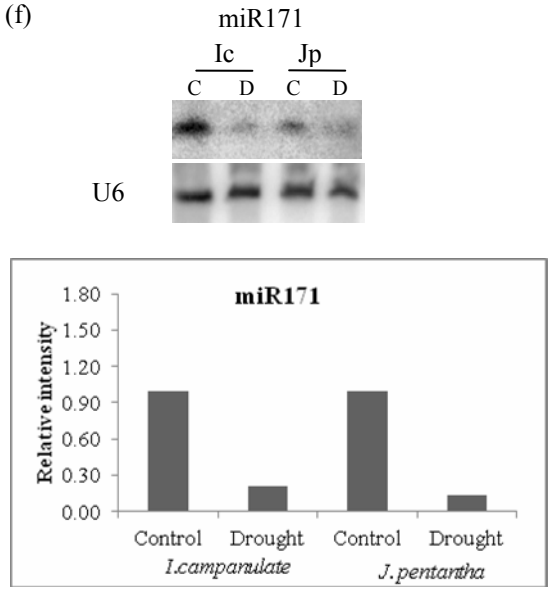
Figure 8 Expression level of conserved miRNAs in *I. campanulata* (Ic) and *J. pentantha* (Jp) leaves growing under *in situ* control (C) and water deficit (D) conditions as analyzed through Northern blotting. U6 (small nuclear RNA) was used as loading control and relative intensities of all miRNAs (to that of control) was quantified by normalizing their intensity values in accordance to that of U6.



(e)


miR168
Ic
Jp
C D C D
U6
miR168
Relative intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control Drought Control Drought
I.camparulate
J. pentantha

(f)


miR171
Ic
Jp
C D C D
U6
miR171
Relative intensity
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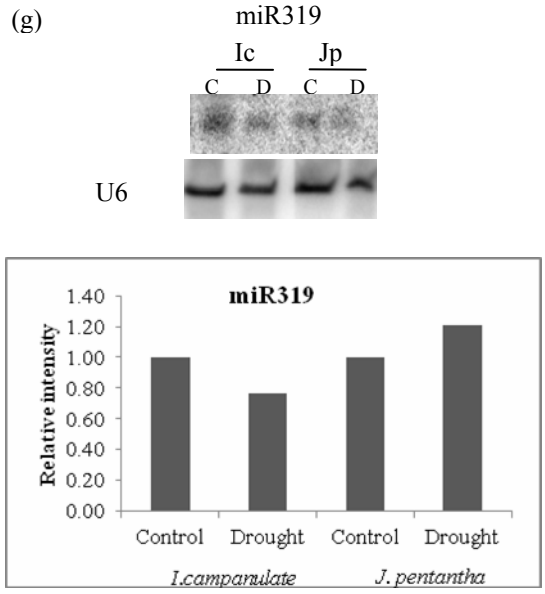
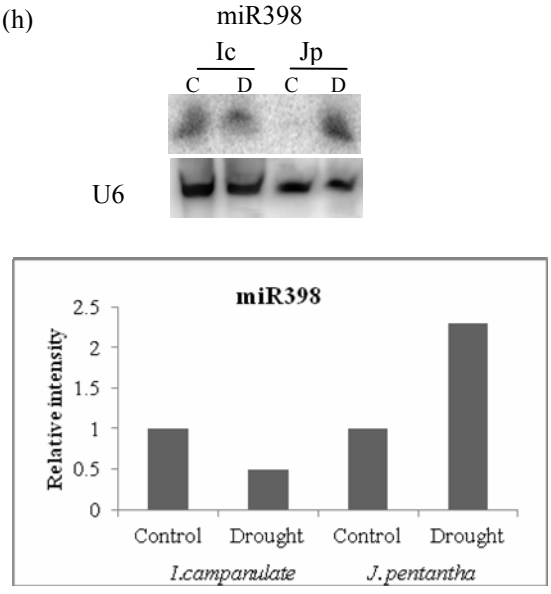
1.50
1.20
0.90
0.60
0.30
0.00
Control Drought Control Drought
I.camparulate
J. pentantha

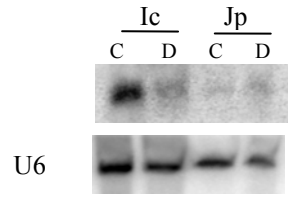
(g)

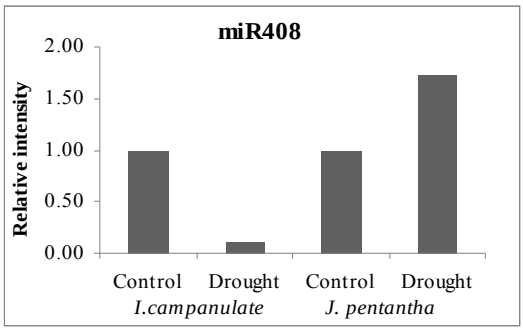

miR319
Ic
Jp
C D C D
U6
miR319
Relative intensity
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control Drought Control Drought
I.camparulate
J. pentantha

(h)

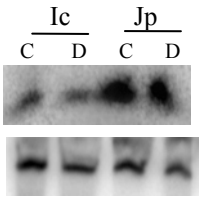

miR398
Ic
Jp
C D C D
U6
miR398
Relative intensity
2.5
2
1.5
1
0.5
0
Control Drought Control Drought
I.camparulate
J. pentantha

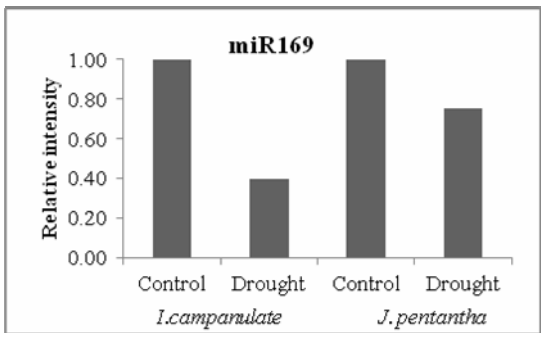
(i) miR408



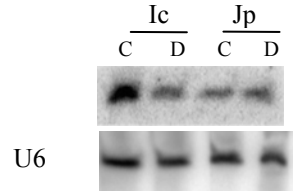



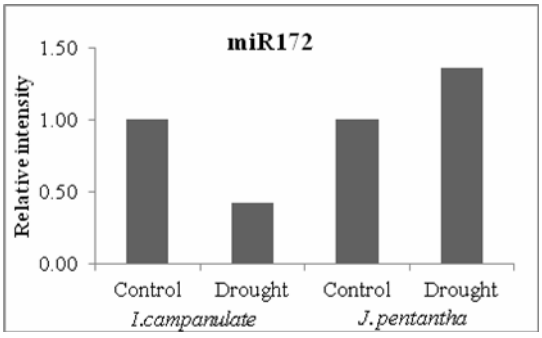

(j) miR169



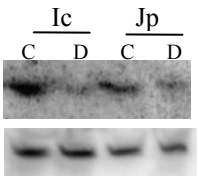



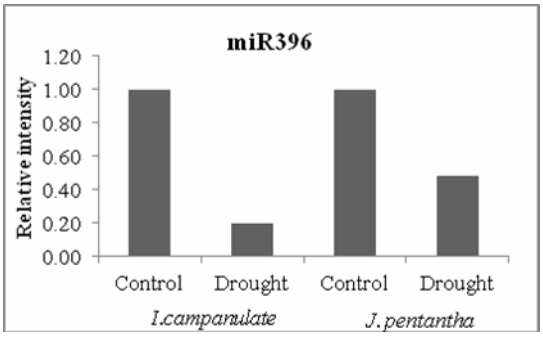

(k) miR172



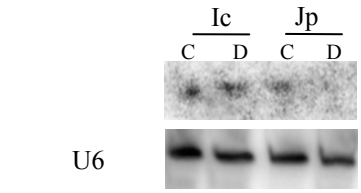



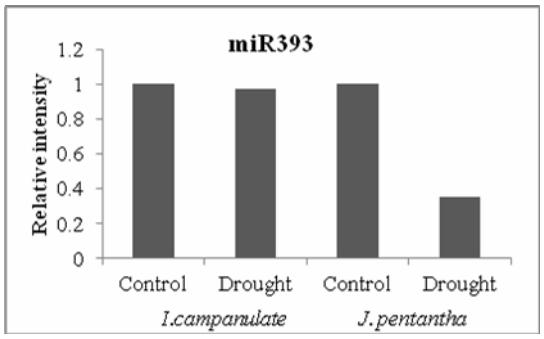

(l) miR396



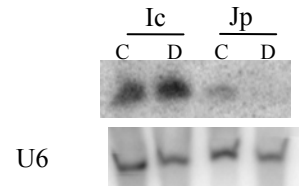



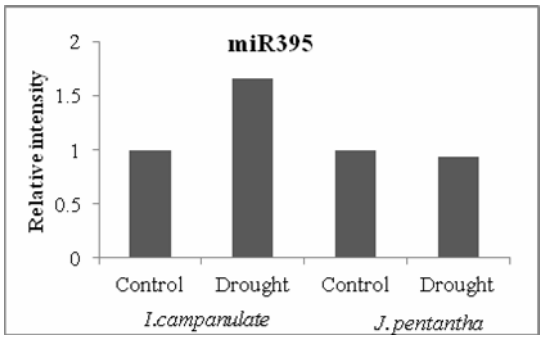
(m) miR393



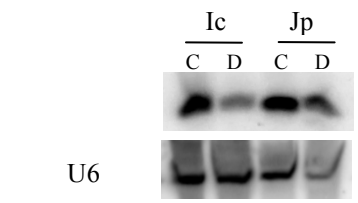



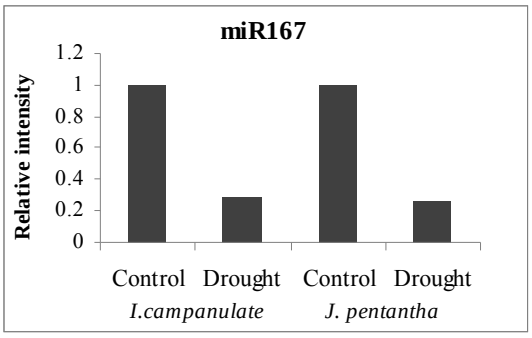

(n) miR395




(o) miR167



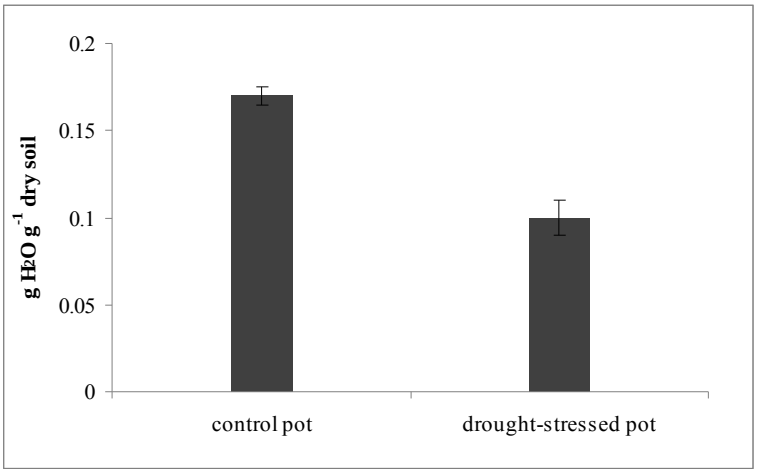
### 3.6. Soil water status in control and drought-stressed pots (*ex situ* conditions)

Under greenhouse (*ex situ*) conditions, drought-stressed pots of both the plant species (*I. campanulata* and *J. pentantha*) were deprived from normal watering schedule for 48h. The control pots were watered according to the normal watering schedule. In order to analyze the effect of water stress on the water content of the soil in the pots, soil water content analyses was carried out. It depicted almost 40% decline in soil water content in the drought-stressed pots than compared to control pots (Figure 9).

### 3.7. Phenotypic variations in *I. campanulata* and *J. pentantha* growing in greenhouse (*ex situ* conditions)

Both the plant species showed symptoms of drought stress symptoms were observed after 48h of water stress. After 48h of stress, *J. pentantha* showed symptoms of leaf wilting (leaf drooping and formation of wrinkles) while minimum effect was seen in *I. campanulata* leaves. At this stage leaf Relative water content (RWC) and SLA of both the species were measured to evaluate the differential response of these species towards water deficit. The leaf RWC of drought stressed *I. campanulata* plants was 40% lesser than the control plants under persisting conditions in our green house. While, leaf RWC of drought stressed *J. pentantha* was 55% lesser than control plants. Results indicate prominent reduction in leaf RWC of *J. pentantha* plants than compared to *I. campanulata* exposed to equivalent conditions of water stress. *I. campanulata* showed reduction in SLA from 291.7$cm^2g^{-1}$ under control to 284.2$cm^2g^{-1}$ under drought stress. *J. pentantha* showed reduction in SLA from 282.5$cm^2g^{-1}$ under control to 276.3$cm^2g^{-1}$ under drought stress. Results indicate minor reduction in SLA in both the species exposed to water stress.

Figure 9 Soil water content analyzed in control and drought-stressed pots. Data are mean ±SD (n=10)




### 3.8. Physiological and biochemical response of *I. campanulata* and *J. pentantha* growing in greenhouse (*ex situ* conditions)

Physiological and biochemical response of *I. campanulata* and *J. pentantha* towards *ex situ* water deficit was examined by analyzing the alterations in selected parameters such as chlorophyll content, anthocyanin concentration, levels of lipid peroxidation and levels of SOD isoforms.

#### 3.8.1. Chlorophyll content

Chlorophyll concentration analyzed in control and drought-stressed pots was observed to be different in both the species. Both the species showed reduction in chlorophyll concentration. The degree of reduction was different in both the species. *I. campanulata* exposed to water deficit showed 13.4% reduction in chlorophyll concentration while *J. pentantha* showed 34.4% reduction in chlorophyll concentration (Table 6). Results portrayed prominent drop in the chlorophyll content in *J. pentantha* than *I. campanulata* under similar levels of water deficit.

### 3.8.2. Anthocyanin content

*I. campanulata* and *J. pentantha* showed increase in the concentration of anthocyanin under water stress than compared to control. Degree of anthocyanin accumulation in response to water deficit varied between the two species. *I. campanulata* showed 56.4% rise in anthocyanin levels; while *J. pentantha* showed a rise of 204.1% (almost 2 fold) as compared to control (Table 6). Results showed that the accumulation of anthocyanin in *J. pentantha* was almost 147% higher than those observed in *I. campanulata*.

### 3.8.3. Lipid peroxidation

Water deficit induced oxidative damage was analyzed through lipid peroxidation assay. Levels of lipid peroxidation were determined by measuring the concentration of MDA. Both the species exposed to *ex situ* water deficit showed rise in the concentration of MDA. However the accumulation of MDA was different in both the species. *I. campanulata* growing under water deficit showed 26.8% rise in concentration of MDA than control; while *J. pentantha* exposed to similar levels of water deficit showed 77.4% rise in concentration of MDA (Table 6).

Table 6 Chlorophyll, anthocyanin and MDA concentration in *I. campanulata* and *J. pentantha* growing under control and drought- stressed pots. Data are ±SD (n=3).

| Parameters | Control pot | | Drought- stressed pot | |
|---|---|---|---|---|
| | *I. campanulata* | *J. pentantha* | *I. campanulata* | *J. pentantha* |
| Chlorophyll mg $g^{-1}$ fresh weight | 16.5±0.52 | 7.2±0.4 | 14.29±0.75 | 4.72±0.63 |
| Anthocyanin ug $g^{-1}$ fresh weight | 0.39±0.05 | 0.97±0.07 | 0.61±0.07 | 2.95±0.09 |
| MDA n mol $g^{-1}$ fresh weight | 12.65±1.24 | 11.95±1.85 | 16.04±1.32 | 21.20±1.5 |

### 3.8.4. Superoxide dismutases

SOD isoforms were identified and their levels were analyzed in both the species growing under *ex situ* conditions. A total of 8 SOD isoforms (2 MnSODs, 2 FeSODs and 4 CuZnSODs) were identified. Band intensities of these SOD isoforms were analyzed using the AlphaEaseFC software. Relative band intensity of all SOD isoforms was calculated for both the species. Both the species, growing under control and water deficit conditions, showed different levels of SOD isoforms. MnSODs I and II showed rise in their activity exposed to water deficit than control (Figure 10a). However, the rise in the activity of both the isoforms was observed to be significant in *I. campanulata* ($P<0.05$) and insignificant in *J. pentantha* ($P>0.05$) (Figure 10b). *I. campanulata* exposed to water stress showed 22.5% and 23.4% rise in the activity of MnSODI and MnSODII respectively than observed under control conditions (Figure 10b). FeSOD isoforms (FeSODI and FeSODII) also showed rise in their activity in both the species exposed to water deficit than compared control (Figure 10a). However, the rise in the activity of these isoforms was observed to be significant in *I. campanulata* ($P<0.05$) and insignificant in *J. pentantha* ($P>0.05$) (Figure 10c). The rise in the activities of FeSODI and FeSODII was observed to be 15.8% and 14.6% respectively in *I. campanulata* exposed to water deficit conditions than compared to control (Figure 10c).

Contrary to the differential activities of MnSOD and FeSOD isoforms between the two species, CuZnSOD isoforms showed significant rise in their activities in *I. campanulata* and *J. pentantha* exposed to water deficit than control ($P<0.05$) (Figure 10a, Figure 10d). The rise in the activity of CuZnSOD isoforms was predominant in *I. campanulata* than *J. pentantha*. Rise in the activities of CuZnSODI and CuZnSODII was 22.5% and 38.7% respectively in *I. campanulata* exposed to water stress (Figure 10d). CuZnSODIII and CuZnSODIV showed almost double the activity levels (i.e. 109% and 121% respectively) in *I. campanulata* under water deficit than compared to control (Figure 10d). *J. pentantha* exposed to water deficit showed rise in the activities of CuZnSODI, CuZnSODII, CuZnSODIII and CuZnSODIV by 4.8%, 20.9%, 17.6% and 10.2% respectively than analyzed under control conditions (Figure 10d).

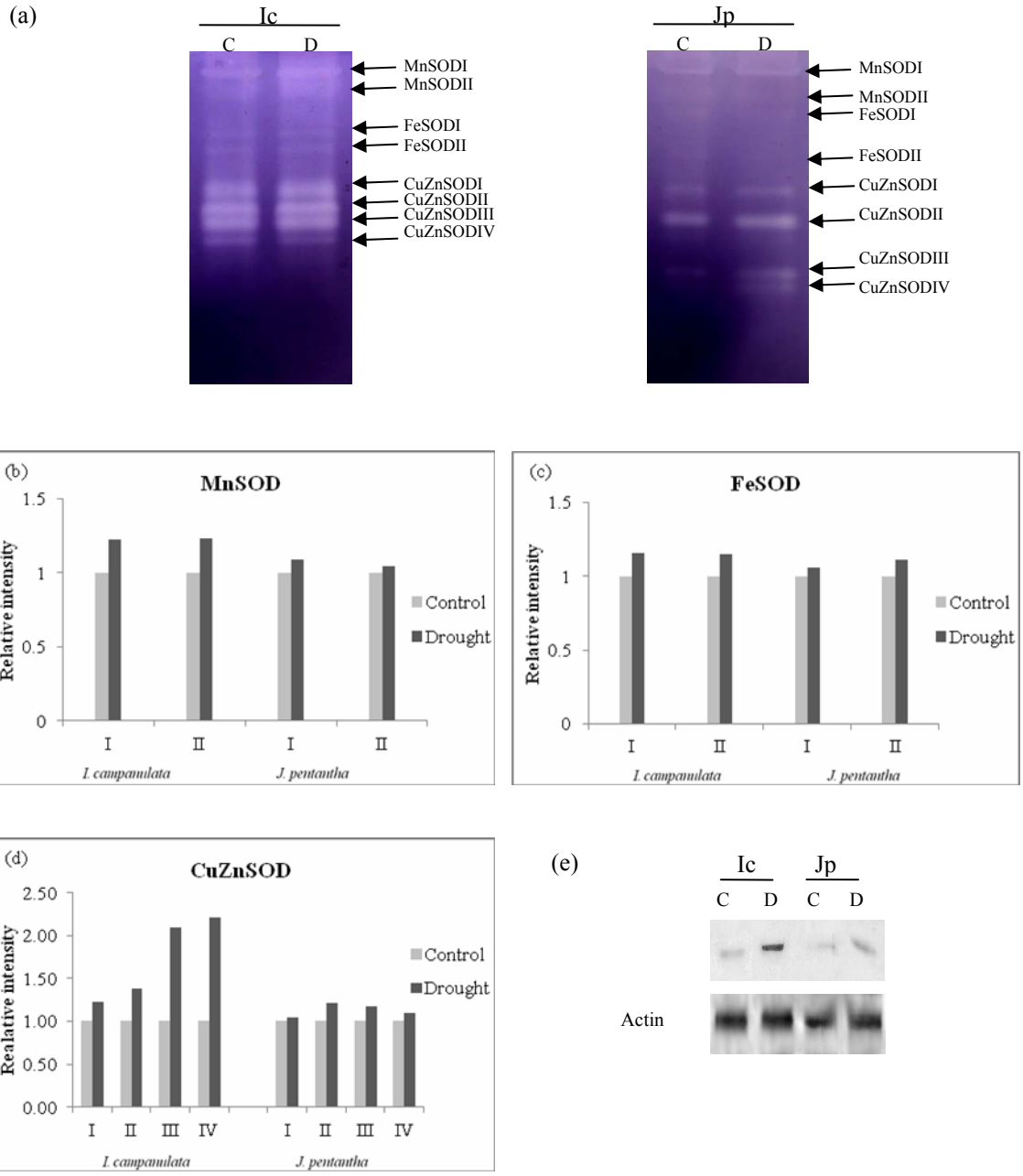
### 3.8.5. Detection of CuZnSODs through immunoblot

For validating the differential induction levels of CuZnSODs in both the species under *ex situ* water deficit, immunoblot analysis for CuZnSOD (CSD2) was performed using polyclonal anti-CSD2 antiserum. Relative band intensities of CuZnSODs were very conspicuous in *I. campanulata* than compared to *J. pentantha* exposed to similar level of water stress (Figure 10e). Results confirmed the differential rise in their activity in both the species towards water deficit as observed in-gel activity assay (Figure 10d, Figure 10e).

### 3.9. Statistical analysis

Statistical analysis evaluated whether the different between measured values of chlorophyll content, anthocyanin concentration, MDA concentration and relative band intensities of SOD isoforms in control and drought stressed plants (*I. campanulata* and *J. pentantha*) were significantly different or not. One way analysis of variance (ANOVA) performed showed that the difference between the values of control and drought stressed plants were significant ($P<0.05$) for chlorophyll content, anthocyanin concentration, MDA concentration and for all the relative band intensity values of SOD isoforms of *I. campanulata* including CuZnSODs of *J. pentantha*. The difference between the relative band intensities values of MnSODs and FeSODs of *J. pentantha* were insignificant ($P>0.05$).

Figure 10 SOD isoforms of *I. campanulata* (Ic) and *J. pentantha* (Jp) growing under *ex situ* control (C) and water deficit conditions (D) as analyzed by (a) activity staining of native PAGE. Relative intensity of (b) MnSOD, (c) FeSOD and (d) CuZnSOD isoforms in *I. campanulata* and *J. pentantha* exposed to water deficit than compared to control. (e) Immunoblot showing level of CuZnSOD in *I. campanulata* and *J. pentantha* growing under control and water deficit conditions. Actin was used as loading control.



### 3.10 Quality of total RNA extracted from *I. campanulata* and *J. pentantha* growing under *ex situ* conditions

Total RNA extracted from both the species growing under control and drought stressed conditions was resolved on 1% Agarose gel. Results showed three distinct bands of 5S, 18S and 28S rRNA. The intensity of 28S rRNAs band was almost double to that of 18S rRNA bands indicating that the extracted RNA was pure. Further the total RNA quality was analysed through NanoDrop™ 1000 Spectrophotometer. The ratio $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ showed a value ranging between 1.8-2.0. These results further confirm good quality of total RNA extracted from both the species.

### 3.10.1. Sequencing of small RNA libraries from *I. campanulata* and *J. pentantha* growing under *ex situ* conditions

#### a) High through-put sequencing of small RNAs

Four small RNA libraries were generated from the total RNA samples of control and drought stressed leaf tissues of *I. campanulata* and *J. pentantha*. They were sequenced using Illumina-high throughput sequencing technology. After sequencing, 7,814,660 and 4,610,207 total small RNA reads depicted by 3,482,433 and 2,010,241 unique small RNA reads were obtained from control and drought stressed libraries of *I. campanulata* respectively (Table 7a). While from *J. pentantha* 4,973,757 and 5,333,042 total small RNA reads represented by 1,343,665 and 2,340,667 unique reads were obtained from the control and drought stressed libraries respectively (Table 7b). These unique small RNA sequences were used for BLASTn search against the known conserved miRNAs of various species available at the miRNA database (miRBase version 21) to identify the precursor miRNAs (pre-miRNAs) and mature miRNAs in both the species. Unique pre-miRNA sequences obtained from control and drought stressed libraries of *I. campanulata* are 5,741 and 4,414 respectively; whereas 4,185 and 5,072 sequences were obtained from *J. pentantha* control and drought libraries respectively (Table 7a and Table 7b). Mature miRNA reads identified from control and drought stressed samples of *I. campanulata* are 2,981 and 2,351 (Table 7a) while 1,755 and 2,217 mature miRNA reads were identified from *J. pentantha* control and drought stressed libraries respectively (Table 7b). The leftover unique sequences were searched for the degradation products of

non-coding RNAs like tRNA, rRNA and snoRNA using the non-coding RNA database. 84,596 and 92,895 are the unique non-coding RNAs identified from *I. campanulata* control and drought stressed libraries respectively (Table 7a). Whereas 112,212 and 85,376 are the unique non-coding RNAs identified from *J. pentantha* control and drought stressed libraries respectively (Table 7b).

The abundance of total unique small RNA reads ranging from 18-30nt length in *I. campanulata* and *J. pentantha* are shown in the Figure 11a and Figure 12a. It portrays that the abundance of these small RNAs at different lengths (18-30nt) is almost similar in both the species. Under control conditions, both the species show the maximum reads at 24nt length followed by second highest reads at 23nt length (Figure 11a, Figure 12a). While under water deficit conditions, the maximum small RNA reads in both the species were observed at 24nt length followed by second highest reads at 21nt length (Figure 11a, Figure 12a). These results depict that, under stress the small RNA reads in both the species were observed to increase at 21nt size class, which largely consist of miRNAs.

The distribution of unique repeat sequences and ncRNA sequences at 18-30nt length in *I. campanulata* control and stressed libraries is shown in Figure 11b and Figure 11c respectively. Figure 12b and Figure 12c shows the size distribution of unique repeat sequences and ncRNA sequences in *J. pentantha* control and stressed libraries respectively. Under control conditions, both the species showed maximum unique repeat sequence reads at 21nt length followed by second and third highest reads at 22nt and 24nt length respectively (Figure 11b, Figure 12b). Even under drought stress, both the species showed maximum reads at 21nt length. However, *I. campanulata*, under drought stress, showed second and third highest reads at 23nt and 22nt length respectively. While *J. pentantha* under drought showed second and third highest reads at 22nt and 23nt length respectively. In response to drought, both the species showed accumulation of repeat sequences at 21nt length; however accumulation was also seen at 23nt length in *I. campanulata.* ncRNA sequences in both the species showed maximum reads at 21nt length under control conditions (Figure 11c, Figure 12c). Under drought stress, almost 5 fold accumulation of ncRNA was seen in both the species.

Abundance of unique pre-miRNA and mature miRNA reads ranging from 18-30nt in control and drought stressed libraries of both the species are shown in Figure 11d, Figure 11e, Figure 12d and Figure 12e. Under control conditions, the maximum pre-miRNA reads in both the species are observed at 24nt length; preceded by the second highest reads at 21nt length (Figure 11d, Figure 12d). Under drought stress the pre-miRNA reads in both the species were predominantly higher at 21nt length (Figure 11d, Figure 12d). Abundance of mature miRNA sequences were maximum at 21nt length followed by second highest peak at 22nt and 24nt length under both control and drought stress in the two Convolvulaceae species (Figure 11e, Figure 12e). Under stress the mature miRNAs reads was observed to increase by almost 4 fold in both the species (Figure 11e, Figure 12e).

**b) Identification of miRNAs**

As genomic information for *I. campanulata* and *J. pentantha* is currently not available, miRNA orthologs or paralogs were identified from unique reads by aligning the miRNA sequences (from both the species) to the miRNA homologs present in the miRNA database (miRBase version 21). A total of 213 and 177 miRNA belonging to 42 families were identified from control and drought stressed libraries of *I. campanulata* respectively. From *J. pentantha*, a total of 150 and 176 miRNAs belonging to 37 families were identified from control and drought stressed libraries respectively. Amongst these miRNA families, 35 miRNA families were identified in both the species. These miRNAs are considered as analogous miRNAs (Table 8a). It includes miR156, miR157, miR159, miR160, miR162, miR164, miR165, miR166, miR167, miR168, miR171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398, miR399, miR403, miR408, miR1310, miR2111, miR2111-5, miR2911, miR482, miR5139, miR530, miR6173, miR6300, miR6478, miR858 and miR894. Many of these miRNAs (miR156, miR157, miR159, miR160, miR162, miR165, miR166, miR167, miR168, miR172, miR319, miR394, miR396 and miR408) were highly expressed in both the control and drought stress libraries of *I. campanulata* and *J. pentantha*. Apart from analogous miRNAs, 7 miRNA families were solely identified in *I. campanulata* while 2 miRNA families were solely identified in *J. pentantha* (Table 8b). These miRNAs are considered as alien miRNAs. This includes miR169, miR447, miR473, miR477, miR4995, miR5141 and miR530-5

as solely detected in *I. campanulata*; and miR414 and miR3630-3 as solely detected in *J. pentantha* (Table 8b). It indicates similarity as well as differences in the miRNA families identified in the two species. These miRNAs showed very low expression in both control and drought stress libraries of both the species.

Many of the miRNAs identified in both the species of Convolvulaceae were ubiquitously identified in model, crop and other species (under comparison); few miRNAs identified amongst the species (under comparison) were unusual (Figure 13). miR156, miR159, miR160, miR162, miR164, miR166, miR167, miR168, miR169, miR170/171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398 and miR399 were ubiquitously identified in *I. campanulata*, *J. pentantha* and all the plant species under comparison (Figure 13). These miRNAs were treated as highly conserved miRNAs. On the contrary, miR3630-3 was identified solely in *J. pentantha* and not observed in any other plant species selected for comparison. miR414 identified solely in *J. pentantha* was previously identified in *A. thaliana* and *O. sativa*, however it was not reported in rest of the species under comparison (Figure 13). miR169 was identified in *I. campanulata* and all the species under comparison, however its expression was not identified in *J. pentantha*. miR447, miR473, miR477, miR530-5, miR4995 and miR5141 were identified solely in *I. campanulata* (i.e. absent in *J. pentantha*) (Figure 13). Amongst these miRNAs, miR447 was previously identified only in *A. thaliana*; miR473 was previously identified in *P. trichocarpa*; while miR477 was previously identified in *P. persica*, *P. trichocarpa* and *S. tuberosum*. miR530-5, miR4995 and miR5141 were identified in all the other species under comparison (Figure 13). It indicated that these miRNAs had specificity in narrow range of plant species.

In order to understand the nucleotide composition at a specific position in all the mature miRNA sequences of *I. campanulata* and *J. pentantha*, position weight matrices (PWMs) was constructed using WebLogo (Crooks et al., 2004). Results of this analysis showed that the 5' end of all the miRNAs examined were 75% U bias based on the graphed nucleotide composition per position. This was same for both the species (Figure 14a, Figure 14b). Other than this, there are several other positions on *I. campanulata* and *J. pentantha* miRNAs that show bias base conservation. For example, position 8 was G bias in both the species; however the frequency was higher in *I. campanulata* miRNAs (45%) than *J. pentantha* miRNAs (40%) (Figure 14a,

Figure 14b). C bias was seen at position 19 in both the species, wherein it was 70% in *J. pentantha* and 60% *I. campanulata*. Similarly, C bias was also seen at position 18 which was comparatively higher in *J. pentantha* than *I. campanulata* (Figure 14a, Figure 14b). Position 5 had higher frequency of A in *J. pentantha* (40%) and *I. campanulata* (35%). 40% G bias was seen at position 3 in both species. Moreover position 2 showed 40% U bias in *I. campanulata* and 35% G bias in *J. pentantha*. Position 17 on mature miRNAs showed 30% A bias in *J. pentantha* and 30% U bias in *I. campanulata*. Position 4 showed higher frequency of G (35%) in *J. pentantha* and A (40%) in *I. campanulata*. Likewise, 40% C and A bias were seen in *J. pentantha* and *I. campanulata* respectively at position 6 (Figure 14a, Figure 14b).

Table 7a Statistics of small RNA sequences/reads from control and drought stressed libraries of *I. campanulata* (Ic)

| Category | Ic control library | | Ic drought stressed library | |
|---|---|---|---|---|
| | Number of unique reads | Number of total reads | Number of unique reads | Number of total reads |
| Known mature miRNAs | 2,981 | 628,066 | 2,351 | 394,931 |
| Known pre-miRNAs | 5,741 | 676,322 | 4,414 | 420,180 |
| ncRNAs | 84,596 | 1,128,955 | 92,895 | 823,379 |
| Repeats | 34,600 | 244,242 | 34,771 | 199,246 |
| Total | 3,482,433 | 7,814,660 | 2,010,241 | 4,610,207 |

Table 7b Statistics of small RNA sequences/reads from control and drought stressed libraries of *J. pentantha* (Jp)

| Category | Jp control library | | Jp drought stressed library | |
|---|---|---|---|---|
| | Number of unique reads | Number of total reads | Number of unique reads | Number of total reads |
| Known mature miRNAs | 1,755 | 682,937 | 2,217 | 557,632 |
| Known pre-miRNAs | 4,185 | 699,640 | 5,072 | 566,222 |
| ncRNAs | 112,212 | 1,971,704 | 85,376 | 980,687 |
| Repeats | 38,727 | 699,137 | 34,360 | 2,38,450 |
| Total | 1,343,665 | 4,973,757 | 2,340,667 | 5,333,042 |

Figure 11a Size distribution of total unique sequences identified from *I. campanulata* control (blue) and drought stressed (red) libraries which are expressed as percentage of unique sequences/reads.

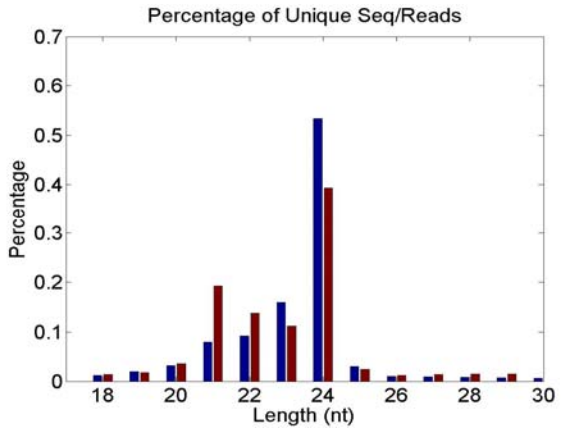



Figure 11b Size Distribution of repeat sequences identified from *I. campanulata* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

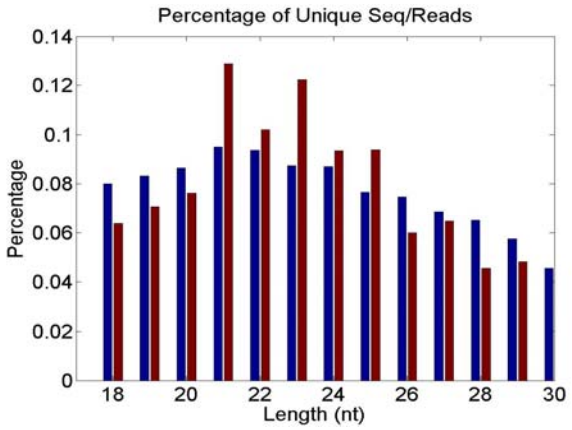


Figure 11c Size distribution of non-coding small RNA (ncRNA) sequences identified from *I. campanulata* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

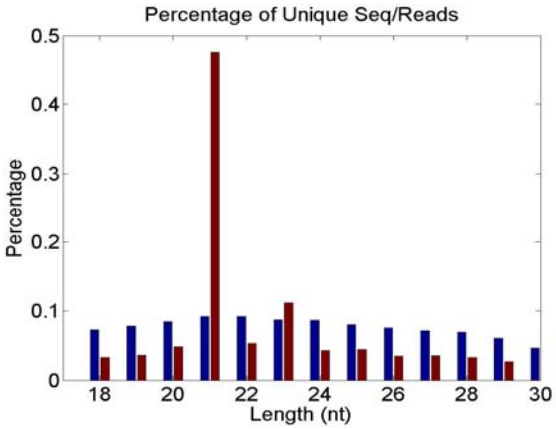



Figure 11d Size distribution of pre-miRNA sequences identified from *I. campanulata* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

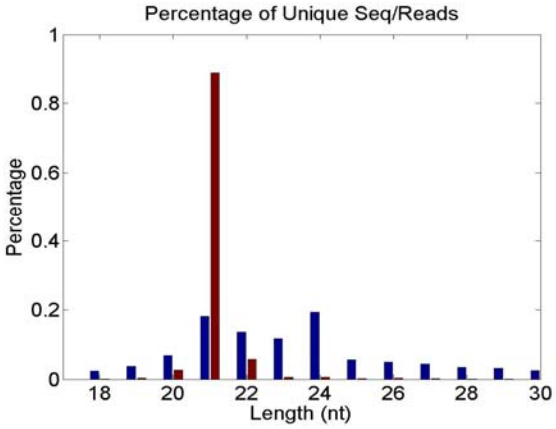


Figure 11e Size distribution of mature miRNA sequences identified from *I. campanulata* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

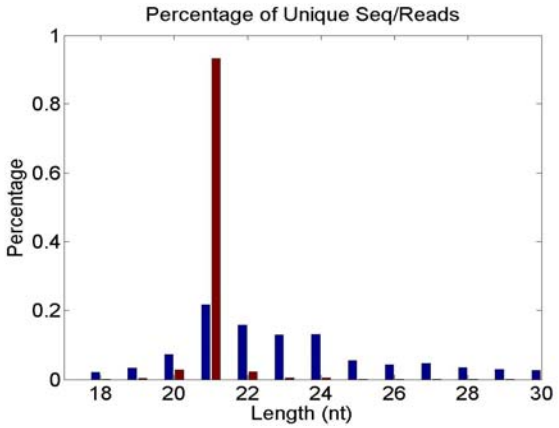



Figure 12a Size distribution of total unique sequences identified from *J. pentantha* control (blue) and drought stressed (red) libraries which are expressed as percentage of unique sequences/read.

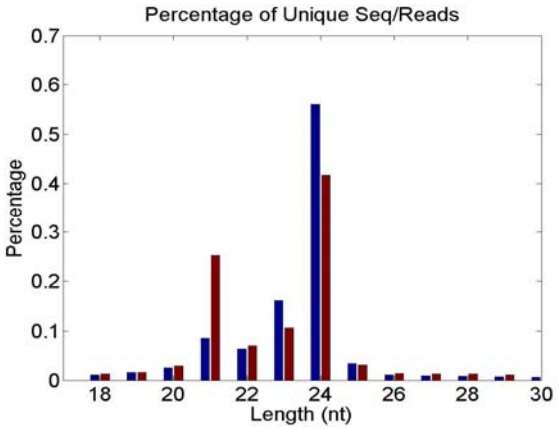


Figure 12b Size Distribution of repeat sequences identified from *J. pentantha* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

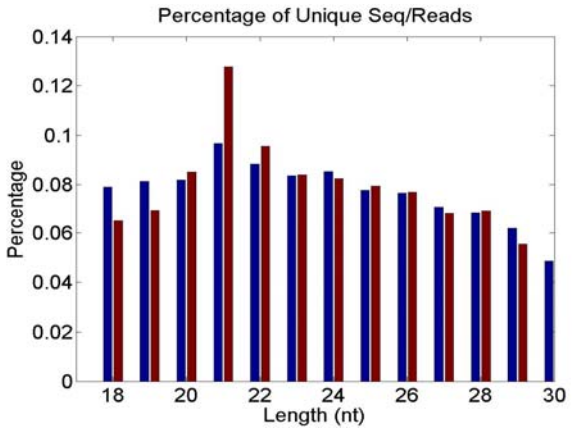



Figure 12c Size distribution of non-coding small RNA (ncRNA) sequences identified from *J. pentantha* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

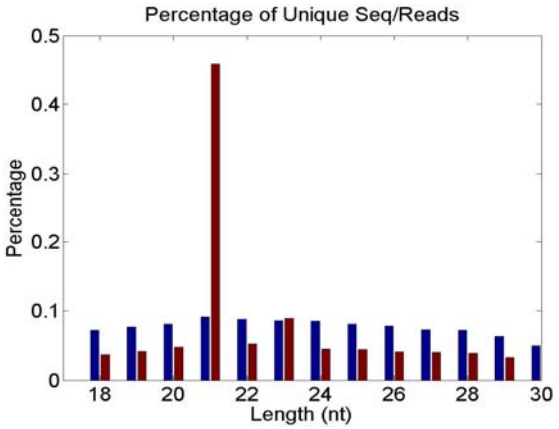


Figure 12d Size distribution of pre-miRNA sequences identified from *J. pentantha* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

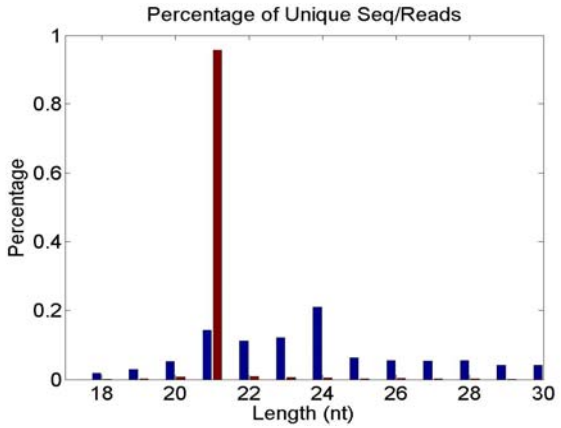



Figure 12e Size distribution of mature miRNA sequences identified from *J. pentantha* control (blue) and drought (red) stressed libraries which are expressed as percentage of unique sequences/reads.

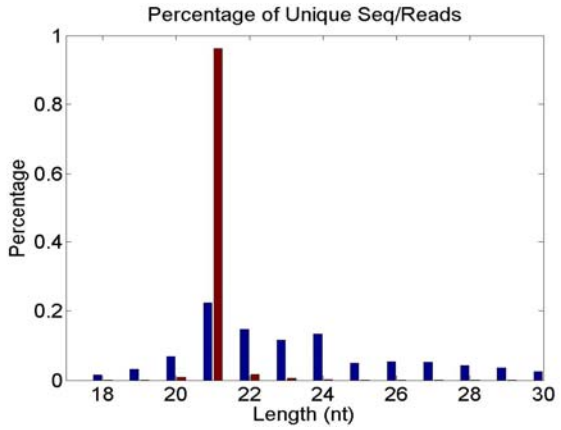


Table 8a Analogous miRNA families identified in the two Convolvulaceae species and their expression

| miRNA families | Normalized miRNA reads (RPTM) of *I. campanulata* | | Normalized miRNA reads (RPTM) of *J. pentantha* | |
|---|---|---|---|---|
| | Control | Water deficit | Control | Water deficit |
| miR156 | 19712 | 18030 | 7807 | 16355 |
| miR157 | 961 | 603 | 1094 | 516 |
| miR159 | 4687 | 4306 | 8694 | 11575 |
| miR160 | 413 | 347 | 141 | 300 |
| miR162 | 1760 | 3525 | 1060 | 1628 |
| miR164 | 394 | 217 | 54 | 377 |
| miR165 | 265 | 275 | 597 | 416 |
| miR166 | 987328 | 1063453 | 2393340 | 1560620 |
| miR167 | 20394 | 12785 | 6215 | 17180 |
| miR168 | 20408 | 73216 | 37636 | 52762 |
| miR171 | 242 | 106 | 12 | 34 |
| miR172 | 2905 | 1740 | 235 | 1693 |
| miR319 | 807 | 833 | 117 | 309 |
| miR390 | 14 | 17 | 432 | 654 |
| miR393 | 41 | 35 | 2 | 39 |
| miR394 | 586 | 234 | 229 | 653 |
| miR395 | 67 | 17 | 32 | 131 |
| miR396 | 227384 | 152399 | 77038 | 192734 |
| miR397 | 3 | 0 | 16 | 0 |
| miR398 | 19 | 121 | 826 | 793 |
| miR399 | 74 | 30 | 54 | 71 |
| miR403 | 58562 | 63151 | 97 | 371 |
| miR408 | 138 | 319 | 3673 | 1873 |
| miR1310 | 36 | 48 | 177 | 129 |
| miR2111 | 10 | 0 | 0 | 6 |
| miR2111-5 | 228 | 226 | 0 | 2 |
| miR2911 | 47 | 37 | 52 | 30 |
| miR482 | 4 | 7 | 0 | 4 |
| miR5139 | 6 | 9 | 0 | 2 |
| miR530 | 22 | 33 | 2 | 0 |
| miR6173 | 26 | 46 | 38 | 26 |
| miR6300 | 3 | 2 | 0 | 4 |
| miR6478 | 49 | 13 | 24 | 38 |
| miR858 | 1574 | 3258 | 0 | 2 |
| miR894 | 1 | 4 | 0 | 4 |

Table 8b Alien miRNA families identified solely in *I. campanulata* or *J. pentantha* and its expression ("-" indicates lack of expression)

| miRNA families | Normalized miRNA reads (RPTM) of *I. campanulata* | | Normalized miRNA reads (RPTM) of *J. pentantha* | |
|---|---|---|---|---|
| | Control | Water deficit | Control | Water deficit |
| miR169 | 22 | 0 | - | - |
| miR447 | 0 | 4 | - | - |
| miR473 | 0 | 4 | - | - |
| miR477 | 9 | 22 | - | - |
| miR4995 | 1 | 0 | - | - |
| miR5141 | 3 | 2 | - | - |
| miR530-5 | 17 | 15 | - | - |
| miR3630-3 | - | - | 0 | 2 |
| miR414 | - | - | 0 | 2 |

Figure 13 Comparing the miRNAs identified in *I. campanulata*, *J. pentantha* and few other species

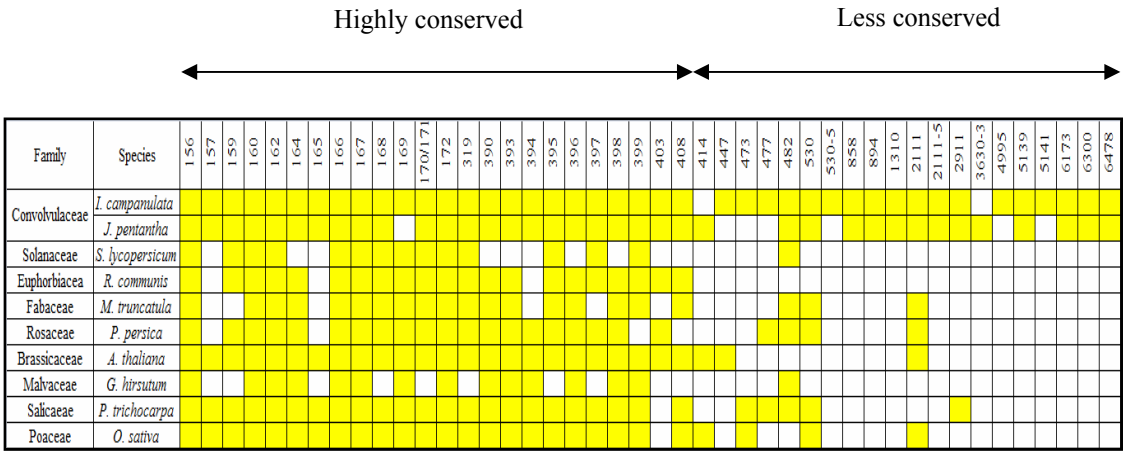
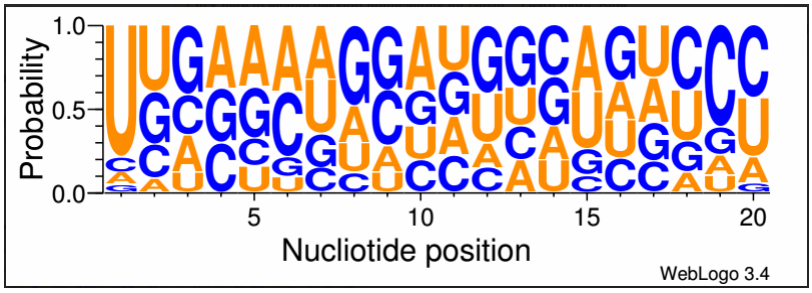
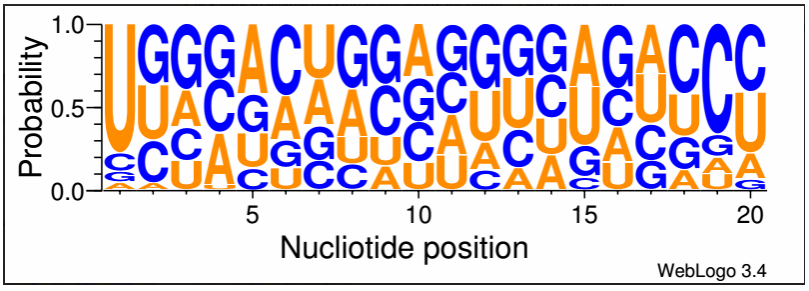



Figure 14 Position weight matrices of (a) *I. campanulata* and (b) *J. pentantha* mature miRNAs

(a)




(b)



### c) Expression of miRNAs identified from *I. campanulata* and *J. pentantha* (control libraries)

After removing the miRNAs whose expression was lower than 100 RPTM, we could identify abundantly expressed miRNAs. It includes 170 miRNA members belonging to 19 miRNA families in *I. campanulata* and 136 miRNA members belonging to 17 miRNA families in *J. pentantha*. Comparing the normalized miRNA expression in the control libraries of the two species showed differences in the abundance of analogous miRNAs. miR166 followed by miR396 are the most abundantly expressed miRNAs in control sample of both the species (Table 9). miR156, miR157, miR159, miR160, miR162, miR164, miR165, miR166, miR167, miR168, miR171, miR172, miR319, miR394, miR396, miR403, miR408, miR858 and miR2111-5 showed higher expression in *I. campanulata* than compared to *J. pentantha* (Table 9). Expression of miR319, miR164, miR172, miR171 and miR403 was 5.89, 6.29, 11.36, 19.16 and 602.73 fold higher in *I. campanulata* than compared to *J. pentantha* respectively. While expression of miR156, miR160, miR167, miR394 and miR396 was 1.56-2.28 fold higher in *I. campanulata* than compared to *J. pentantha*. Interestingly, expression of miR858 and miR2111-5 was only detected in the control library of *I. campanulata* and not from *J. pentantha* control libraries (Table 9). *J. pentantha* control samples showed higher expression of miR157, miR159, miR165, miR166, miR168, miR390, miR408 and miR1310 than compared to *I. campanulata* (Table 9). Expression of miR398, miR408 and miR390 was 42.47, 25.62 and 29.86 fold higher in *J. pentantha* than *I. campanulata* respectively. While expression of miR165, miR166 and miR1310 was 1.25-3.92 fold higher in *J. pentantha* than *I. campanulata*. These results portray differential expression of analogous conserved miRNAs between the species. Targets of these miRNAs are also conserved amongst *Arabidopsis* and several other plant species. Based on the identification and conservation of miRNA-target in *Arabidopsis*, putative miRNA targets and their functional annotation are listed in Table 9. Differences in expression of these miRNAs between the species may provide them characteristic signature for growth, development and stress response.

Table 9 Abundantly expressed miRNAs in *I. campanulata*, *J. pentantha* or either of the control libraries; and their conserved putative targets and functional annotation as identified in *Arabidopsis*

| miRNA families | Normalized miRNA reads (RPTM) | | Putative conserved Target | Functional annotation |
|---|---|---|---|---|
| | *I. campanulata* control library | *J. pentantha* control library | | |
| miR156 | 19712 | 7807 | SBP-like TF | Flowering and anthocyanin accumulation |
| miR157 | 961 | 1094 | SBP-like TF | Shoot development and flowering |
| miR159 | 4687 | 8694 | MYB TF | Heading time |
| miR160 | 413 | 141 | ARF-10, 16,17 | Organogenesis; embryogenesis |
| miR162 | 1760 | 1060 | DCL1 | Regulates expression of other miRNAs and development |
| miR164 | 394 | 54 | CUC1 and CUC2 | Plant growth |
| miR165 | 265 | 597 | leucine-zipper HD-ZIP III | Apical meristem formation; organ polarity; vascular development |
| miR166 | 987328 | 2393340 | | |
| miR167 | 20394 | 6215 | ARF-6,8 | Gynoecium and stamen development (fertility) |
| miR168 | 20408 | 37636 | AGO-1 | Regulates miRNA pathways |
| miR171 | 242 | 12 | GRAS/ Scarecrow-like protein-TF | Axillary meristem differentiation |
| miR172 | 2905 | 235 | APETALA2-like TF | Flower development and time |
| miR319 | 807 | 117 | TCP-TF | Cell proliferation-leaf and petal size |

| | | | | |
|---|---|---|---|---|
| miR390 | 14 | 432 | AGO7 | tasiRNA biogenesis |
| miR394 | 586 | 229 | SKP1-Cullin/CDC53-F-box | Leaf morphology (curling) |
| miR396 | 227384 | 77038 | GRF | Leaf growth and development |
| miR398 | 19 | 826 | CSD1, CSD2, CCS | Oxidative stress |
| miR403 | 58562 | 97 | AGO1/AGO2 | Responsive to viral infection |
| miR408 | 138 | 3673 | copper protein-plantacyanin; laccase | Copper homeostasis |
| miR1310 | 36 | 177 | EPS (epi-cedrol synthase) | Lyase activity |
| miR2111-5 | 228 | 0 | Kelch domain-containing F-box protein | Unknown |
| miR858 | 1574 | 0 | MYB family genes | Fruit development |

### d) Response of *I. campanulata* miRNAs to drought

From the abundantly expressed miRNAs (i.e. expression > 100 RPTM), drought associated miRNAs were identified by comparing the normalized expression of miRNAs in drought stressed and control libraries of *I. campanulata* (Figure 15a). Eleven conserved miRNAs (miR156, miR157, miR159, miR160, miR164, miR167, miR171, miR172, miR394, miR396, miR2111-5) showed decrease in its expression in response to drought stress. While nine other miRNAs (miR162, miR165, miR166, miR168, miR319, miR403, miR398, miR408 and miR858) showed induction in their expression towards drought stress. Amongst these miRNAs, those showing greater than or equal to 0.5 fold change in their expression in response to water stress were classified as drought responsive miRNAs in this study. Results of this analysis revealed 12 drought responsive miRNAs, wherein 7 miRNAs were downregulated and 5 miRNAs were upregulated (Table 10a). Amongst the downregulated miRNAs, major downregulation was shown by miR394 (1.50 fold) followed by miR171 (1.28 fold) (Table 10a). Whereas other miRNAs including miR164, miR172, miR167, miR157 and miR396 showed reduction in their expression by 0.82, 0.67, 0.60, 0.59

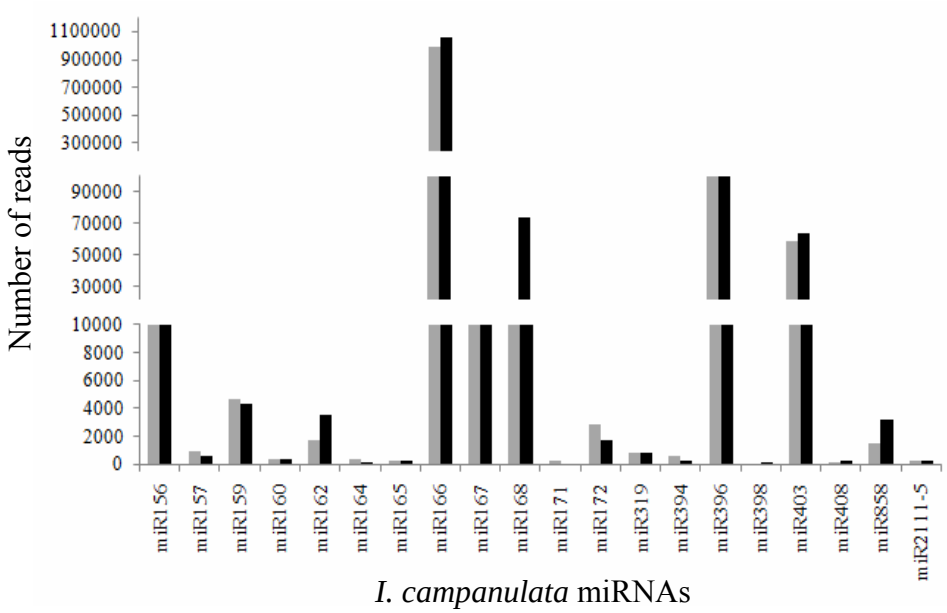
and 0.50 fold respectively in response to water stress. Amongst the upregulated miRNAs, miR398 showed major induction in its expression (5.37 fold). Followed by this, miR168, miR408, miR858 and miR162 showed increase in their levels by 2.59, 1.31, 1.07 and 1.00 fold respectively in reaction to water deficit. Results revealed that several abundantly expressed miRNAs were downregulated while few were upregulated by water stress in *I. campanulata*.

**e) Response of *J. pentantha* miRNAs to drought**

In *J. pentantha*, several abundantly expressed (> 100 RPTM) miRNAs ( miR156, miR159, miR160, miR162, miR164, miR167, miR168, miR172, miR319, miR390, miR394, miR395, miR396 and miR403) were upregulated in response to water deficit; while few others (including miR157, miR165, miR166, miR398, miR408 and miR1310) were downregulated (Figure 15b). Similar to *I. campanulata*, miRNAs showing a fold change equal to or greater than 0.5 fold were considered as drought responsive miRNAs. Result of the analysis showed drought mediated upregulation of 12 miRNAs and downregulation of 3 miRNAs in *J. pentantha* (Table 10b). miRNAs showing major upregulation in response to drought stress are miR164, miR172, miR393, miR395 and miR403 having an increase in its expression by 5.98, 6.20, 18.50, 3.09 and 2.82 fold respectively (Table 10b). Other than these, miRNAs such as miR156, miR160, miR167, miR171, miR319, miR394 and miR396 showed increase in its expression by 1.09, 1.13, 1.76, 1.83, 1.64, 1.85 and 1.50 fold respectively under influence of water stress. On the contrary, major reduction in expression was showed by miR157 (1.12 fold) in *J. pentantha* under water stress. While miR166 and miR408 showed reduction in its expression by 0.53 and 0.96 fold respectively under water deficit (Table 10b). Results showed that majority of abundantly expressed miRNAs were upregulated in *J. pentantha* growing under water deficit.

Figure 15 Comparing the expression of miRNAs identified in control and drought stressed libraries of (a) *I. campanulata* (b) *J. pentantha*

(a)




(b)

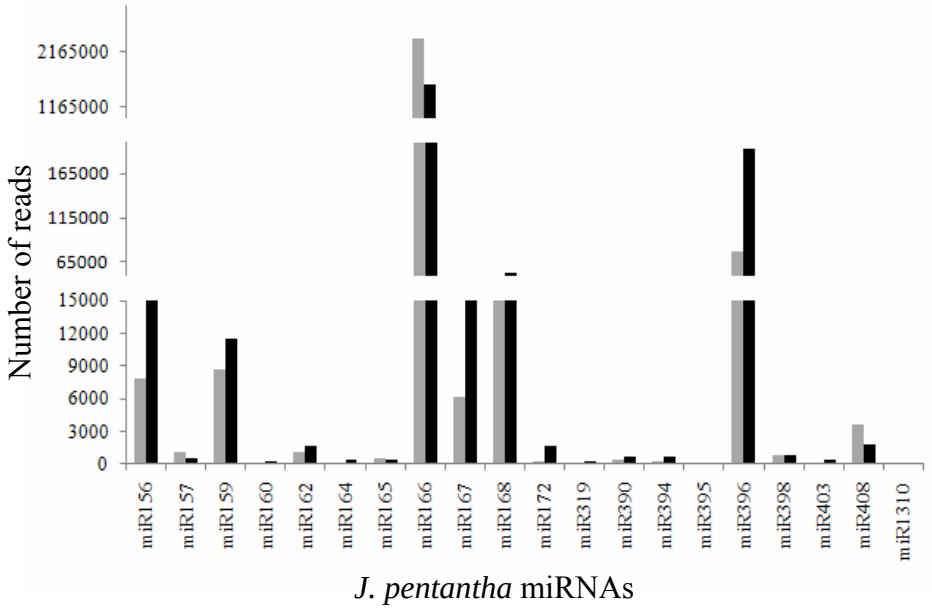


Table 10 Drought responsive miRNAs identified in (a) *I. campanulata* and (b) *J. pentantha*

(a)

| miRNA family | Expression pattern | fold change |
|---|---|---|
| miR157 | downregulated | 0.59 |
| miR164 | | 0.82 |
| miR167 | | 0.60 |
| miR171 | | 1.28 |
| miR172 | | 0.67 |
| miR394 | | 1.50 |
| miR396 | | 0.50 |
| miR162 | upregulated | 1.00 |
| miR168 | | 2.59 |
| miR398 | | 5.37 |
| miR408 | | 1.31 |
| miR858 | | 1.07 |

(b)

| miRNA families | Expression pattern | fold change |
|---|---|---|
| miR157 | downregulated | 1.12 |
| miR166 | | 0.53 |
| miR408 | | 0.96 |
| miR156 | upregulated | 1.09 |
| miR160 | | 1.13 |
| miR162 | | 0.54 |
| miR164 | | 5.98 |
| miR167 | | 1.76 |
| miR172 | | 6.20 |
| miR319 | | 1.64 |
| miR390 | | 0.51 |
| miR394 | | 1.85 |
| miR395 | | 3.09 |
| miR396 | | 1.50 |
| miR403 | | 2.82 |

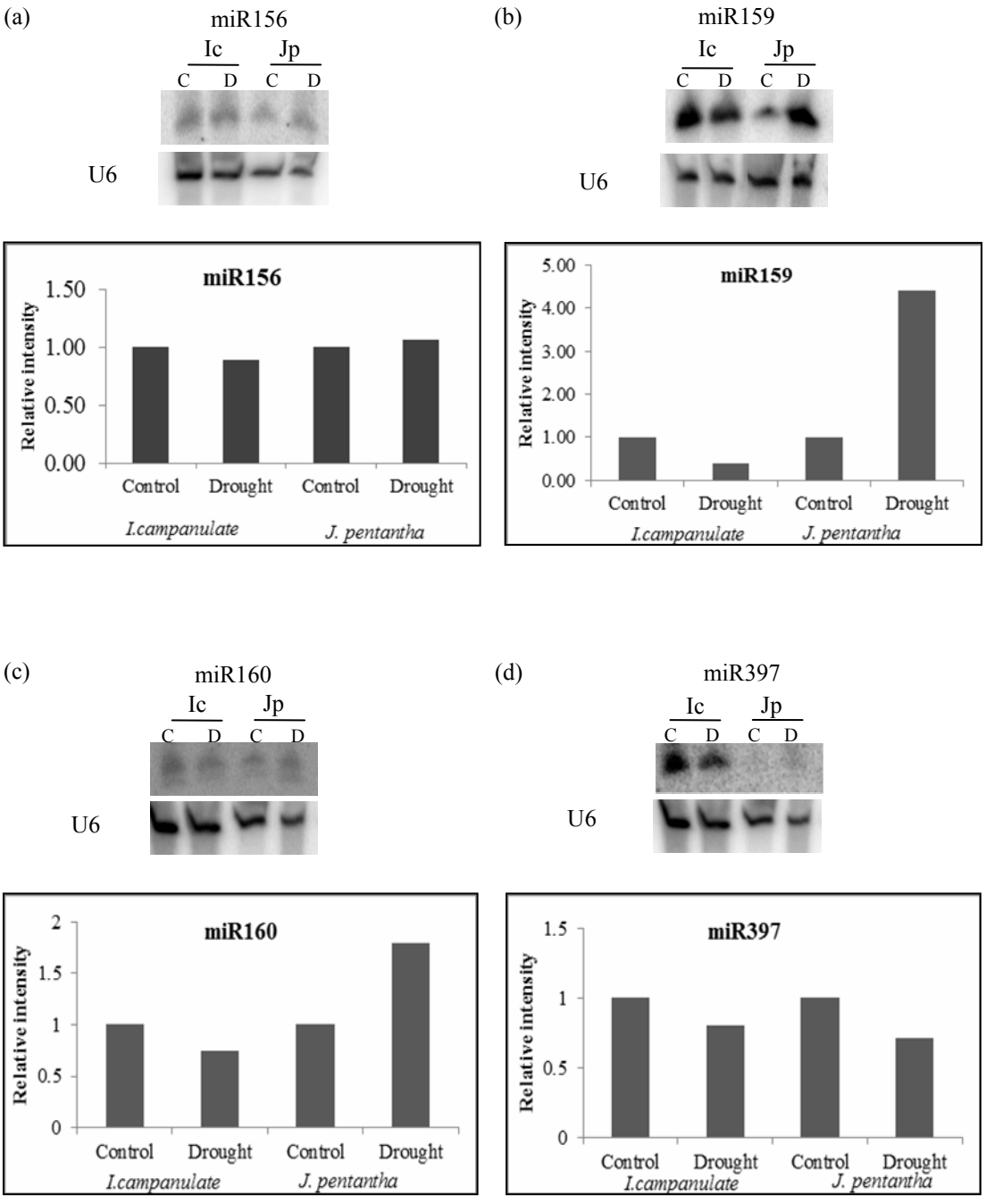
### 3.10.2 miRNA blot analysis of *I. campanulata* and *J. pentantha* growing under *ex situ* conditions

In order to validate the expression of few of the highly conserved miRNA analysed through small RNA sequencing, Northern blotting was carried out. The blots were probed for conserved miRNA sequences of *Arabidopsis* which were end labeled with $\gamma$-$^{32}$P-ATP. The images acquired by scanning the phosphor screen with Typhoon scanner were loaded into the software and band intensities were obtained. Each of the band intensities were normalized in accordance to U6 (small nuclear RNA), that was used as loading control. The relative band intensities (i.e. band intensity of water deficit samples to that of control) for each conserved miRNA under consideration were analysed and calculated. Blot analysis in *I. campanulata* revealed that majority of miRNAs (amongst the selected miRNAs) showed reduction in their expression under drought stress than compared under control conditions (Figure 16). miR156, miR159, miR160, miR397, miR171, miR169, miR172, miR396, miR393, miR395 and miR167 showed reduction in their expression in response to water stress. Amongst these downregulated miRNAs, miR159 showed major reduction (1.50 fold) in its expression (Figure 16b). Successively, miR167, miR396 and miR169 showed reduction in their levels by 1.47, 0.98 and 0.88 fold respectively (Figure 16o, Figure 16l, Figure 16j). Whereas other miRNAs including miR156, miR160, miR397, miR171, miR172, miR393 and miR395 showed marginal reduction in their levels (0.13-0.65 fold) under water stress (Figure 16a, Figure 16c, Figure 16d, Figure 16f, Figure 16k, Figure 16m, Figure 16n). On the contrary, miR319, miR168, miR398 and miR408 showed increase in their levels (0.12-1.8 fold) under conditions of water scarcity (Figure 16g, Figure 16e, Figure 16h, Figure 16i). Overall the results portrayed that expression pattern (i.e. either downregulation or upregulation) of these miRNAs of *I. campanulata* as analysed through Northern blot were comparable to those observed by sequencing.

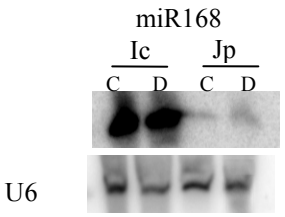
Northern blot profiling of *J. pentantha* samples revealed that majority of miRNAs (under consideration) were upregulated in drought stressed samples when compared to control once. miR156, miR159, miR160, miR168, miR171, miR319, miR169, miR393, miR395 and miR167 showed induction in their levels in response to drought stress (Figure 16a, Figure 16b, Figure 16c, Figure 16e, Figure 16f, Figure 16g, Figure 16j, Figure 16m, Figure 16n, Figure 16o). Amongst these miRNAs, miR393 showed

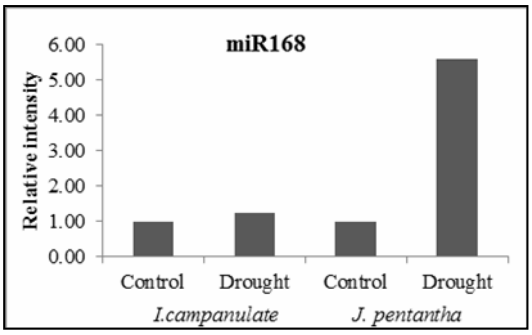
increase in its levels by 28.77 fold (Figure 16m). Followed by it, miR168, miR395, miR167 and miR159 showed induction in their expression by 4.60, 4.04, 3.49 and 3.40 fold respectively under stress (Figure 16e, Figure 16n, Figure 16o, Figure 16b). In response to stress, miR319, miR171, miR169 and miR160 showed increase in their levels by 1.65, 1.04, 0.95 and 0.80 fold respectively (Figure 16h, Figure 16g, Figure 16f, Figure 16j, Figure 16c). miR156 showed marginal induction (0.07 fold) in its levels under stress (Figure 16a). On the other hand, miR397, miR398, miR408, miR172 and miR396 showed decrease in their expression under drought stress (Figure 16d, Figure 16h, Figure 16i, Figure 16k, Figure 16l). Amongst these miRNAs, miR396 showed maximum downregulation (0.53 fold) under stress (Figure 16l). miR397, miR398, miR408 and miR172 showed minor downregulation under stress (Figure 16d, Figure 16h, Figure 16i, Figure 16k). These results suggested that the expression pattern (i.e. either upregulation or downregulation) of most of the *J. pentantha* miRNAs as analysed through Northern blotting were largely comparable to that observed through sequencing. However expression pattern of miR172 and miR396 were not similar to those analysed through sequencing. The discrepancy in expression pattern of these miRNAs cannot be ascribed to differences in plant material (from which RNA was extracted) as same RNA source was used for blot and sequencing profiles. It may be attributed to the biased-ligation with the adapters in the sequencing technique. In addition expression of miR169 was not detected by sequence analysis due to low expression; however blot analyses showed upregulation by 0.95 fold.

Figure 16 Expression level of conserved miRNAs in *I. campanulata* (Ic) and *J.pentantha* (Jp) leaves growing under Control (C) and Drought (D) conditions in greenhouse as analysed through Northern blotting. U6 (small nuclear RNA) was used as loading control and relative accumulation of all miRNAs (to that of Control) was quantified by normalizing their intensity values in accordance to that of U6.

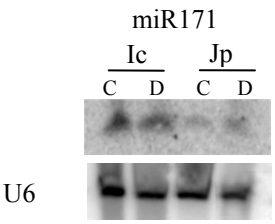


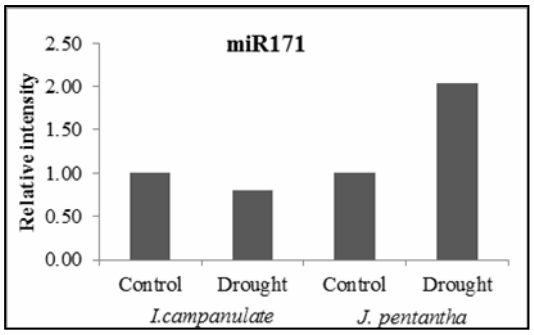
(e)


miR168
Ic
Jp
C D C D
U6

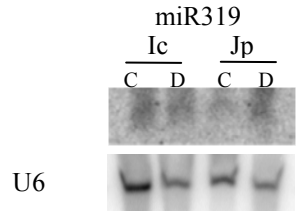

miR168
Relative intensity
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

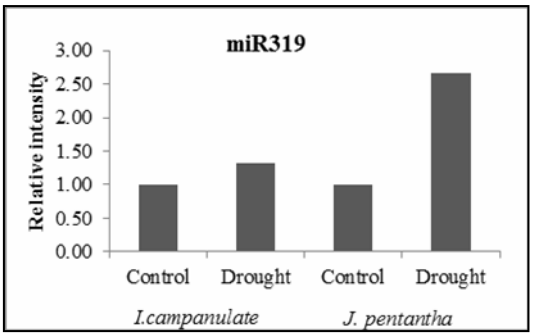
(f)


miR171
Ic
Jp
C D C D
U6

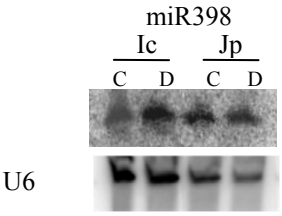

miR171
Relative intensity
2.50
2.00
1.50
1.00
0.50
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

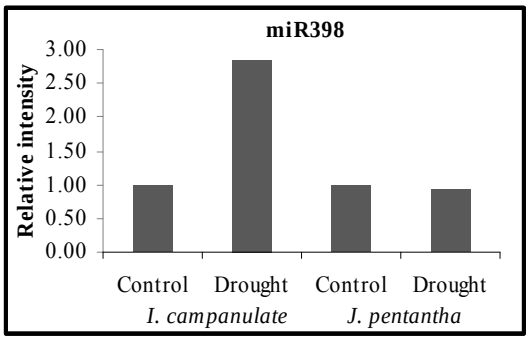
(g


miR319
Ic
Jp
C D C D
U6

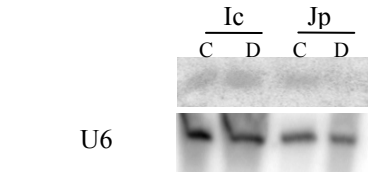

miR319
Relative intensity
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

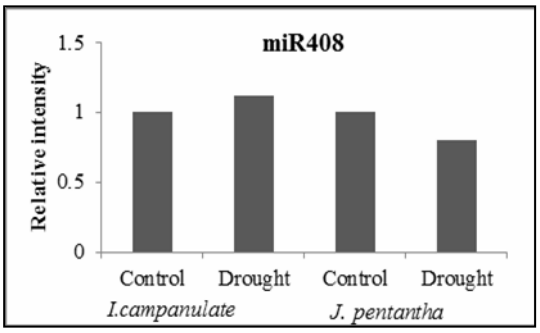
(h


miR398
Ic
Jp
C D C D
U6

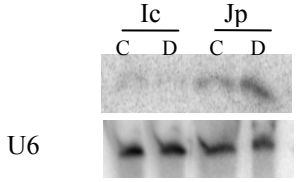

miR398
Relative intensity
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Control
Drought
Control
Drought
I. campanulate
J. pentantha

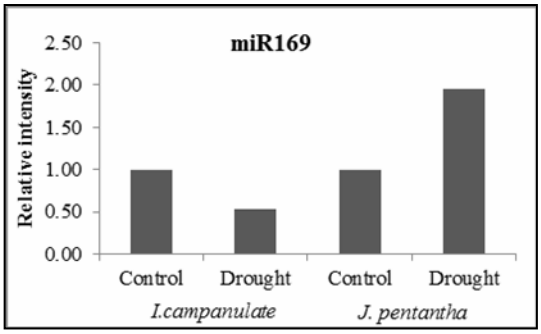
(i)


miR408
Ic
Jp
C D C D
U6

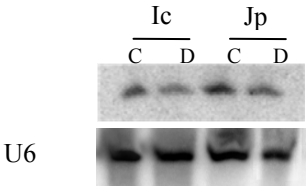

miR408
Relative intensity
1.5
1
0.5
0
Control
Drought
Control
Drought
I.campanulate
J. pentantha

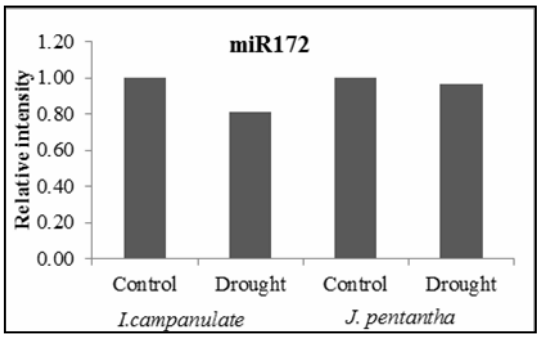
(j)


miR169
Ic
Jp
C D C D
U6

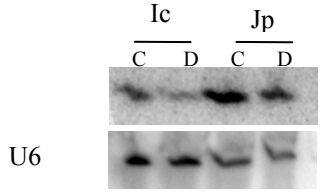

miR169
Relative intensity
2.50
2.00
1.50
1.00
0.50
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

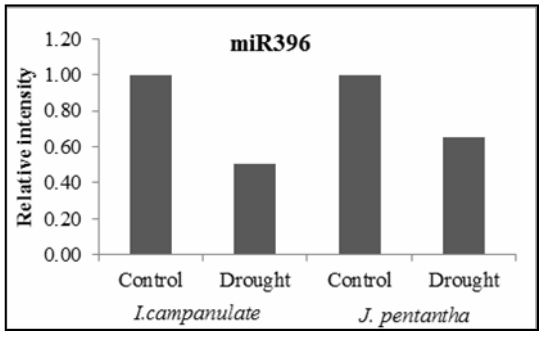
(k)


miR172
Ic
Jp
C D C D
U6

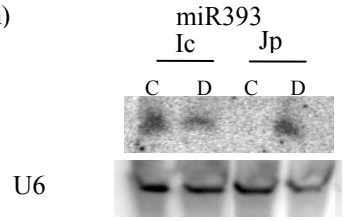

miR172
Relative intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

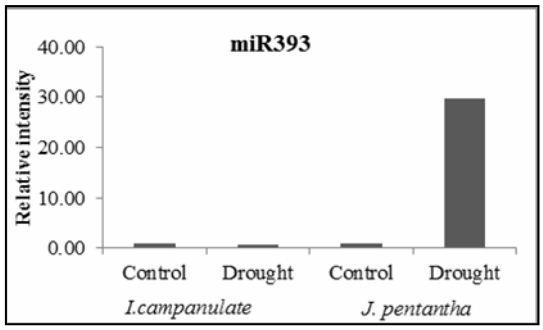
(l)


miR396
Ic
Jp
C D C D
U6

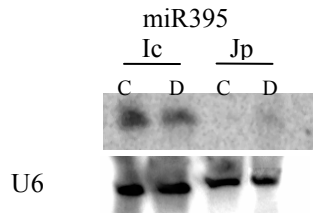

miR396
Relative intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

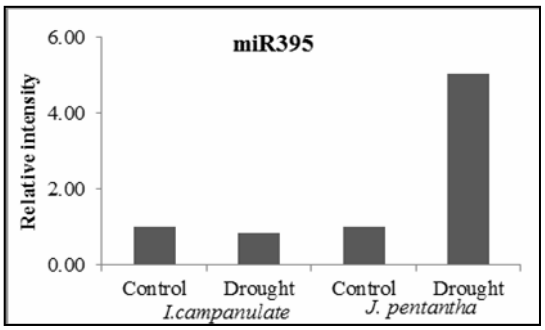
(m)


miR393
Ic
Jp
C D C D
U6

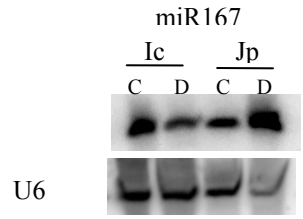

miR393
Relative intensity
40.00
30.00
20.00
10.00
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

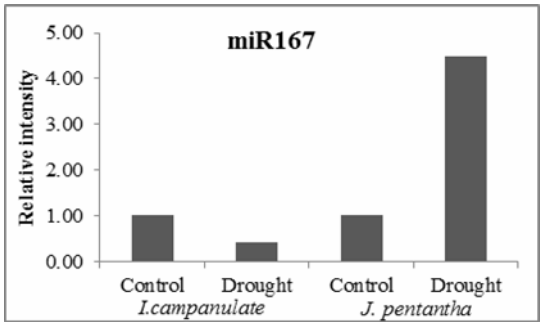
(n)


miR395
Ic
Jp
C D C D
U6


miR395
Relative intensity
6.00
4.00
2.00
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha

(o)


miR167
Ic
Jp
C D C D
U6


miR167
Relative intensity
5.00
4.00
3.00
2.00
1.00
0.00
Control
Drought
Control
Drought
I.campanulate
J. pentantha