# Better root:shoot ratio conferred enhanced harvest index in transgenic groundnut overexpressing the *rd29A:DREB1A* gene under intermittent drought stress in an outdoor lysimetric dry-down trial

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## Abstract

An outdoor confined trial was conducted during the postrainy season of 2009 for physiological evaluation of induced drought tolerance in transgenic plants of groundnut variety JL 24 overexpressing a transcription factor, *DREB1A* driven by the stress-inducible promoter of the *rd29A* gene, both from *Arabidopsis thaliana*. Lysimetric system was used for growing the plants, where intermittent drought stress was imposed at mid-flowering and peak pod-filling stages of the crop, by subjecting plants to a cycle of drying and re-watering. The lysimetric system facilitated complete recovery of roots, thereby, facilitating studies on variations in the root:shoot ratio induced across the genotypes under controlled well-watered (WW) and imposed drought stress (DS) conditions. Under DS the root:shoot ratio showed a significant ($P$ <0.005) positive correlation with pod yield and harvest index (HI), reflecting clearly the better performance of two transgenic events GNRD11 and GNRD33 than the untransformed variety JL 24. The transgenic event GNRD11, in particular, showed enhanced HI along with significantly higher ($P$ <0.05) seed yield that was 22% and 25% higher than JL 24 and the elite breeding groundnut cultivar ICGV 86031, respectively. Better HI in these transgenic events, when compared to the untransformed control, was mainly due to the effective partitioning of the accumulated biomass, more towards roots and pods while relatively less towards shoot biomass, leading to higher root:shoot ratio and better yield, also suggesting better water use efficiency in the former compared to the latter.

## Introduction

Groundnut (*Arachis hypogaea*), an annual legume, is a valuable cash crop for millions of small-scale farmers living mostly in the semi-arid tropics. It is the world's fourth most important source of edible oil, third most important source of vegetable protein and thirteenth most important food crop of the world (Source: http://www.cgiar.org/impact/research/Groundnut.html). The crop is grown in 25.2 million ha throughout the world in over 100 countries between the latitudes of 40° N and 40° S with a total global production of 36.5 million tons (Source: http://fastat.fao.org, 2008). Besides various biotic constraints (Sharma and Ortiz 2000), the productivity of groundnut is mainly limited by water deficits as the crop is frequently subjected to drought stresses of different duration and intensities (Reddy et al. 2003) causing over 6.7 million tons loss in world groundnut production (Subbarao et al. 1995), equivalent to over US$ 520 million, annually (Sharma and Lavanya 2002). Thus, genetic improvement of groundnut for enhancing drought tolerance becomes essential for developing drought-tolerant varieties that can produce higher yields under water-limited conditions.

Highly conserved genome with very low polymorphism (Varshney et al. 2005) and the genetic isolation of the tetraploid (or amphidiploid) groundnut from its wild diploid ancestors, has lead to limited success in the genetic improvement of cultivated groundnuts through conventional and marker-assisted breeding methods (Sharma and Ortiz 2000). Due to the scarcity of available water in the semi-arid tropical regions, drought management strategies, whether agronomic or genetic, need to focus on maximizing the extraction of available soil moisture and the efficiency of its use in crop establishment including growth, biomass and grain yield (Serraj et al. 2005). Genetic engineering has been used to complement traditional breeding methods in crop improvement at ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), as an attempt to generate additional genetic diversity that is

not available in the existing germplasm (Gupta and Varshney 2005, Varshney et al. 2005, Ravi et al. 2011). A transcription factor, *DREB1A* driven by the stress inducible promoter of the *rd29A* gene, both from *A. thaliana* was used for the genetic transformation of a drought-sensitive groundnut variety, JL 24 (Bhatnagar-Mathur et al. 2007). Six of the initially raised 50 transgenic events, having single transgene inserts were selected based on differences in their respective transpiration efficiency (TE) observed during preliminary pot-based dry-down experiments (Bhatnagar-Mathur et al. 2004). In a previous work carried out in contained greenhouse conditions (Vadez et al. 2007), it was also found that the overexpression of *DREB1A* in these transgenic groundnut events increased water uptake under terminal drought stress conditions by an enhanced rooting that resulted in an increase of the root:shoot ratio under water deficit conditions. However, the stress imposed was terminal and severe as well as the study did not intend to assess the effect of water extraction differences on yield. Present work was initiated to assess and evaluate the correlation of the induced changes in root:shoot ratio in the selected transgenic groundnut events with their pod and seed yield under the imposed intermittent drought in field conditions using the lysimetric dry-down procedure.

## Material and methods

### Plant material

Transgenic plants ($T_7$ generation) from the previously selected six groundnut transgenic events, viz, GNRD2, GNRD11, GNRD12, GNRD19, GNRD20 and GNRD33, having single copy of the *DREB1A gene* (Bhatnagar-Mathur et al. 2007), along with controls, viz, the untransformed parent JL 24, and ICGV 86031, a drought tolerant elite cultivar of groundnut were used to carry out the confined outdoor trial.

### Methodology

Soil mixture for the experiment was prepared by mixing dry alfisol and farmyard manure (FYM) in the ratio of 10:1 (soil:FYM). The soil was previously sieved on a 2-mm mesh to homogenize the soil particle size and ensure that all the lysimetric cylinders would be packed at a similar soil bulk density (Vadez et al. 2008). This mixture was then amended with single super phosphate and muriated potash at 300 mg $kg^{-1}$ soil mixture, each. A lysimetric system (Vadez et al. 2008), that included PVC pipes, or cylinders, measuring 1.2 m long and 20 cm diameter (Fig. 1a), providing a surface area and soil volume close to the field conditions, was used for conducting the dry-down experiment. The cylinders were laid vertically next to one another in a randomized complete block design (RCBD) and the plants were grown at a spacing (25 plants $m^{-2}$) similar to the field conditions. Soil mixture was filled in two equal increments in these lysimeters, each followed by saturation with water (20% W/W) immediately, allowing time for excess water to drain, thereby, wetting the soil profile uniformly. Packing was avoided while filling the soil; further soil was added until the soil surface was 5 cm from the top of the tube. The overall bulk density across the tubes was homogenous and close to the standard value of 1.4 for alfisol. The soil surface of the cylinders was mulched with 2 cm thick layer of polythene beads for minimizing evaporative loss of water. It was shown previously that the beads mulch prevented about 90% of soil evaporation, and subsequent cylinder weights provided a measure of water loss solely through transpiration.

Seeds from the above-mentioned genotypes were grown in the PVC lysimeters. Nine replicates of each genotype with two treatments sets – Drought stressed (DS) and Well-watered (WW) – were planted in mid-January 2009. Initially, two seeds per cylinder were sown; PCR screening-based thinning was done for the transgenic events within three weeks from the date of sowing, leaving one PCR positive plant per cylinder. Simultaneously, thinning of the cylinders with control plants was also done by manually removing the extra plant, leaving one healthy and uniform plant per cylinder. Two rows of the untransformed groundnut variety JL 24, followed by another 2–3 rows of sorghum (*Sorghum bicolor*) were planted simultaneously, outside, adjacent to the net-house as border crops as per the biosafety guidelines of the Government of India. Cylinders were watered regularly to keep them near their field capacity, WW conditions, and the germinated seedlings were uniformly maintained till flowering. Soon after the initiation of flowering that showed little variation amongst the genotypes, ie, 40 days from the date of sowing, saturation of the soil profile of all the cylinders was done by adding 2 L of water in each. Excess water was allowed to seep out for 24 h, following which all the cylinders were weighed using an electronic balance (Mettler®, Geneva, Switzerland) connected with a pulley using a moving stand (Fig. 1b) for recording the initial weights of all the cylinders. The weights were again recorded after 48 h to ascertain the end of water seepage from the cylinders; thus this second recording was taken as the initial stable weight of each cylinder that was referred as the field capacity and starting point of the dry-down experiment. Hereafter, the cylinders were regularly weighed on a weekly basis for monitoring the pattern of

**Figure 1.** Experimental setup – confined outdoor trial using the lysimeters: (a) two sets of cylinders arranged according to RCBD design in a net enclosure; and (b) weighing balance and pulley.

water uptake. The WW plants were maintained close to field capacity by replenishing the water loss due to transpiration. To avoid unaccountable water loss through drainage, re-watering was done when weight of the cylinder went below 1 L from its initial saturated weight. The DS plants were allowed to gradually deplete most of the stored water. The intermittent stress was relieved by re-watering 1500 ml (equivalent to 50 mm rain) at 92$^{nd}$, 97$^{th}$ and 115$^{th}$ days after saturation (DAS). The decision to re-water plants was based on the scoring of leaf wilting on a scale of 1 to 5 where 1 indicated no wilting and 5 represented permanent wilting. Plants were re-watered when a majority of the plants showed wilting symptoms in most of their leaves early in the afternoon with a score of 3. Leaf samples were collected at this point for RNA isolations to be used for confirming the induction and overexpression of *DREB1A* gene in the transgenic events and compared to the untransformed control genotypes, through RT-PCR (reverse transcriptase polymerase chain reaction) analysis, as reported earlier (Bhatnagar-Mathur et al. 2007). The experiment was terminated at crop maturity followed by separation of the shoots and pods from the harvested plants for drying and further recording of their dry-weights.

The roots were extracted gently by washing the soil, layer by layer from both ends of the cylinders, after removing the end caps (Fig. 2), bagged separately and dried in a hot air oven at 80°C. The dry-weights of shoot, root and pods were then used to compute TE along with

**Figure 2.** Complete root extraction from the lysimeters: (a) washing away soil from both ends; (b) most of the soil washed off; and (c) complete root extracted along the length of the cylinder.

the cumulative transpiration (T), which is the sum of transpiration value recorded throughout the experiment. Although the transpiration measurement started at 40 DAS only, we assumed that biomass differences between genotypes were small at 40 DAS and that TE could be calculated and used to compare the genotype based on the total plant weight at maturity, without considering biomass at the time transpiration measurement started. Harvest index (HI) and yield efficiency (Y) were also recorded as described earlier (Bhatnagar-Mathur et al. 2007). Individual plant yield data from the dry-down experiment was analyzed for simple ANOVA using Genstat release-10 software, while correlation between the yield component traits studied were drawn using the statistical analytical program SAS version 9.0.

## Climatic conditions

During the contained field trial, average daily temperature was around 32°C, with minimum night temperature of 21°C and maximum day temperature of 45°C while daily average relative humidity recorded was around 37%, minimum being as low as 18% during the day and maximum reaching 60% at night (Fig. 3).

## Results and discussion

For most effective usage of water, three prerequisites include, the ability to maximize water uptake (transpiration), efficient conversion of the water absorbed into biomass (ie, TE), and finally proficient partitioning of the accumulated biomass favorably towards the harvestable yield product such as grain (ie, HI), expressed symbolically as, Y = T*TE*HI (Passioura 1977). Pod yield, according to this equation is a function of *cumulative transpiration* (T; water lost through the plant canopy), *transpiration efficiency* (TE; biomass produced per unit of water transpired) and *harvest index* (HI; the ratio of grain yield to standing biomass). Significant variation in total water uptake, accumulated biomass and harvestable pod yield was seen among all the tested genotypes, both under WW and DS conditions (Tables 1 and 2) while the DS treatment in general induced reduction of the biomass, pod yield and total water extracted by 44%, 64% and 47% respectively, across the genotypes.

### Well-watered treatment

Under WW conditions, all the components of yield architecture including T, TE and HI were positively and significantly ($P$ <0.001) correlated with each other, as the higher water uptake leading to higher biomass (pod,

shoot and root) accumulation that in turn leading to higher harvestable yield (pod and seed). While the absolute dry-weights of the accumulated root and shoot biomass were positively correlated ($P$ <0.001), their ratio (root:shoot) had no correlation with any of the yield components under WW conditions. Transgenic events RD33, RD20 and RD2 had significantly higher ($P$ <0.05) T than the untransformed control JL 24, which also resulted in a significantly higher ($P$ <0.05) pod yield. Rest of the transgenic events had similar water uptake and pod yield as JL 24. Except the transgenic event RD11 that had significantly lower ($P$ <0.05) TE than the untransformed control, JL24, no significant difference was found in TE among the genotypes studied. The HI was similar across the genotypes under study, with the exception of the transgenic event RD2 that had significantly higher HI when compared to JL 24 (Table 1). The root:shoot ratio was remarkably similar across the transgenic events and controls under WW conditions.

**DS treatment**

Under the imposed intermittent drought stress, similar amounts of water were captured for biomass production. Differences in the proportionate partitioning of the accumulated biomass into shoot, root and pod lead to the variation in the correlation among the component traits of yield. Yield was found to be significantly and positively correlated with T and HI and root:shoot ratio, but had no correlation with TE, while it was negatively correlated with shoot biomass. Root:shoot ratio was also significantly and positively correlated with HI ($r^2$ = 0.46) and pod yield ($r^2$ = 0.75) (Fig. 4). The plants avoid stress by different strategies that include deep rooting (Lopes and Reynolds 2010), reduced leaf area resulting into lower shoot biomass and mechanisms related to increased water use efficiency (Araus et al. 2002). Transgenic events, RD11 and RD33 had relatively higher pod yield with significantly higher seed number and seed dry-weight while having lower shoot dry-weight than the untransformed controls of JL 24 and ICGV 86031. The transgenic event RD11 was unique in having significantly higher root:shoot ratio and HI than JL 24, while it had the lowest biomass accumulated with lowest TE value than the rest of the genotypes. The other better performing transgenic event, RD33, had a high HI and root:shoot ratio that was similar to the event RD11, although its TE was significantly higher ($P$ <0.05) than RD11 but similar

**Table 1. Postharvest dry-weights (n=9; mean ± SD) of the biomass and yield across six transgenic events RD2, RD11, RD12, RD19, RD20 and RD33 and the control untransformed parent JL 24 and ICGV 86031 in the confined outdoor lysimetric trial during postrainy season, 2009 at ICRISAT[1].**

| Genotype | Pod wt (g plant$^{-1}$) | Seed wt (g plant$^{-1}$) | Shoot wt (g plant$^{-1}$) | Root wt (g plant$^{-1}$) | Biomass (g plant$^{-1}$) |
|---|---|---|---|---|---|
| **Drought stressed** | | | | | |
| ICGV 86031 | 7.23$^{abc}$ | 3.34$^{cd}$ | 21$^{bcd}$ | 5.48$^{ab}$ | 32.74$^{cd}$ |
| JL 24 | 7.42$^{abc}$ | 4.18$^{c}$ | 23$^{ab}$ | 5.96$^{ab}$ | 36.22$^{ab}$ |
| RD11 | 9.04$^{a}$ | 6.37$^{a}$ | 16.41$^{e}$ | 5.23$^{bc}$ | 31.16$^{d}$ |
| RD12 | 5.25$^{d}$ | 2.00$^{d}$ | 23$^{abc}$ | 4.72$^{c}$ | 32.65$^{cd}$ |
| RD19 | 6.33$^{cd}$ | 4.58$^{bc}$ | 19.21$^{d}$ | 4.61$^{c}$ | 31.3$^{d}$ |
| RD2 | 6.82$^{bcd}$ | 3.69$^{c}$ | 19.74$^{d}$ | 3.73$^{d}$ | 30.38$^{d}$ |
| RD20 | 6.58$^{cd}$ | 3.7$^{c}$ | 25$^{a}$ | 6.13$^{a}$ | 38.5$^{a}$ |
| RD33 | 8.59$^{ab}$ | 5.85$^{ab}$ | 20.69$^{cd}$ | 6.12$^{a}$ | 35.26$^{bc}$ |
| LSD 5% | 1.833 | 1.374 | 2.47 | 0.756 | 2.74 |
| Grand mean | 7.16 | 4.21 | 21.02 | 5.25 | 33.53 |
| **Well-watered** | | | | | |
| ICGV 86031 | 26.49$^{a}$ | 18.15$^{a}$ | 33.8$^{abc}$ | 5.19$^{bc}$ | 64.63$^{ab}$ |
| JL 24 | 16.59$^{c}$ | 10.88$^{b}$ | 28.9$^{bcd}$ | 4.65$^{cd}$ | 50.12$^{cd}$ |
| RD11 | 15.25$^{c}$ | 9.49$^{b}$ | 27.57$^{cd}$ | 3.94$^{cd}$ | 46.78$^{d}$ |
| RD12 | 17.86$^{bc}$ | 10.64$^{b}$ | 24.32$^{d}$ | 3.44$^{d}$ | 43.47$^{d}$ |
| RD19 | 17.07$^{c}$ | 12.03$^{b}$ | 33.6$^{abc}$ | 6.66$^{ab}$ | 57.38$^{bc}$ |
| RD2 | 22.96$^{ab}$ | 16.84$^{a}$ | 30.5$^{abcd}$ | 4.31$^{cd}$ | 57.55$^{bc}$ |
| RD20 | 26.28$^{a}$ | 18.98$^{a}$ | 36.18$^{ab}$ | 5.5$^{abc}$ | 70.01$^{a}$ |
| RD33 | 24.62$^{a}$ | 18.74$^{a}$ | 37.57$^{a}$ | 6.69$^{a}$ | 69.09$^{a}$ |
| LSD 5% | 5.701 | 4.657 | 7.131 | 1.542 | 10.660 |
| Grand mean | 20.890 | 14.47 | 31.568 | 5.047 | 57.379 |

1. Values followed by the same letter(s) in a column are not significantly different at 5% level ($P$ <0.05).

to that in the control JL 24. In the transgenic events RD11 and RD33 the accumulated biomass was partitioned more effectively towards roots and pods while relatively less towards shoot biomass, leading to higher root:shoot ratio and better yield than JL 24 under DS (Table 3) that resulted into higher HI and root:shoot ratio than the rest. RT-PCR studies involving expression of the *npt*II and *DREB1A* genes (Bhatnagar-Mathur et al. 2007) in the transgenic events was determined in individual plants. The *npt*II gene was constitutively expressed in all the plants, both WW and water stressed, while expression of the *DREB1A* gene was observed only in the water stressed plants (Fig. 5).

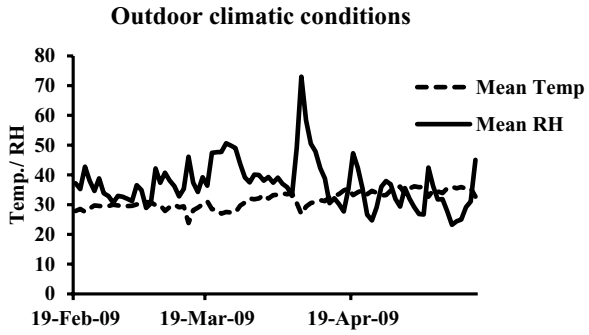


**Figure 3.** Mean temperatures and relative humidity recorded in the confined field, RCW17A, trial during postrainy season, 2009 at ICRISAT.

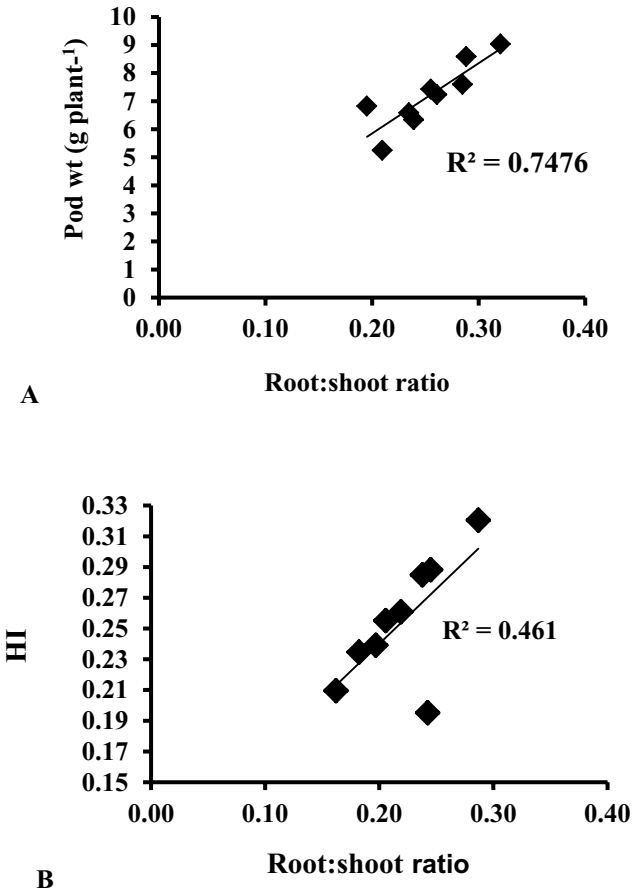


**Figure 4.** Graphical representation of the significantly positive correlation of root:shoot ratio with (A) pod weight and (B) harvest index (HI) as recorded under DS conditions across the six transgenic events and the untransformed variety JL 24 in the outdoor lysimetric dry-down trial conducted during March 4 to May 15, 2009.

**Table 2. Yield components and root:shoot ratio measured (n=9; mean ± SD) across six transgenic events RD2, RD11, RD12, RD19, RD20 and RD33 and the control untransformed parent JL 24 and ICGV 86031 in the confined outdoor lysimetric trial, during postrainy season, 2009 at ICRISAT[1].**

| | HI | | TE | | Root:shoot ratio | |
|---|---|---|---|---|---|---|
| Genotype | WW | DS | WW | DS | WW | DS |
| ICGV 86031 | 0.403$^{a}$ | 0.219$^{bc}$ | 1.64$^{abcd}$ | 1.87$^{de}$ | 0.152$^{abc}$ | 0.261$^{bc}$ |
| JL 24 | 0.313$^{bc}$ | 0.206$^{bcd}$ | 1.67$^{abc}$ | 2.1$^{ab}$ | 0.17$^{abc}$ | 0.255$^{bc}$ |
| RD11 | 0.303$^{c}$ | 0.287$^{a}$ | 1.44$^{d}$ | 1.77$^{e}$ | 0.119$^{c}$ | 0.32$^{a}$ |
| RD12 | 0.37$^{ab}$ | 0.162$^{d}$ | 1.65$^{abcd}$ | 1.94$^{cd}$ | 0.15$^{abc}$ | 0.21$^{de}$ |
| RD19 | 0.299$^{c}$ | 0.197$^{bcd}$ | 1.56$^{cd}$ | 1.79$^{de}$ | 0.200$^{a}$ | 0.239$^{cd}$ |
| RD2 | 0.396$^{a}$ | 0.242$^{ab}$ | 1.60$^{bcd}$ | 1.81$^{de}$ | 0.128$^{bc}$ | 0.195$^{e}$ |
| RD20 | 0.374$^{ab}$ | 0.18$^{cd}$ | 1.86$^{a}$ | 2.2$^{a}$ | 0.113$^{c}$ | 0.235$^{cde}$ |
| RD33 | 0.356$^{abc}$ | 0.245$^{ab}$ | 1.79$^{ab}$ | 2.02$^{bc}$ | 0.187$^{ab}$ | 0.288$^{ab}$ |
| LSD 5% | 0.062 | 0.054 | 0.214 | 0.15 | 0.064 | 0.041 |
| Grand mean | | | | | 0.153 | 0.25 |

1. Values followed by the same letter(s) in a column are not signiûcantly different at 5% level ($P$ <0.05).

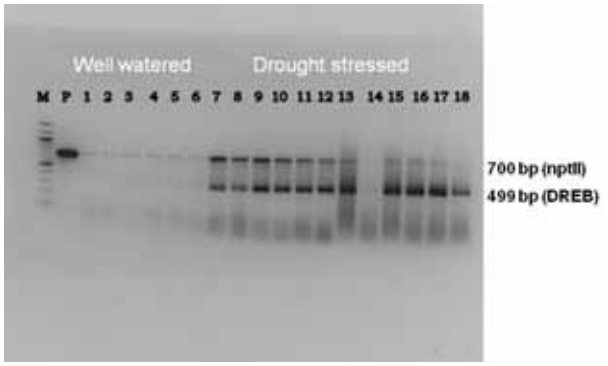


**Figure 5.** RT-PCR analysis: Lane 1 – Plasmid; Lanes 2–6 – Samples collected from WW plants; Lanes 7–18 – Drought stressed samples collected from transgenic plants as well as in transgenic plants under water stress (amplification of *DREB1A* was showing only in transgenic plants under water stress, indicating that induction of rd29A promoter was only under water stress, and not in irrigated conditions); and Lane M – 100bp DNA size marker.

**Table 3. Biomass partitioning pattern observed (n=9; mean ± SD) across six transgenic events RD2, RD11, RD12, RD19, RD20 and RD33 along with the control untransformed parent JL 24 and ICGV 86031 at the end of the confined field, RCW17A, trial during postrainy season, 2009 at ICRISAT[1].**

| Genotype | Shoot (%) | Pod (%) |
|---|---|---|
| ICGV 86031 | 74.8$^{bc}$ | 25.2$^{bc}$ |
| JL 24 | 76.9$^{ab}$ | 23.1$^{cd}$ |
| RD11 | 65.5$^{d}$ | 34.5$^{a}$ |
| RD12 | 81.1$^{a}$ | 18.9$^{d}$ |
| RD19 | 75.2$^{abc}$ | 24.8$^{bcd}$ |
| RD2 | 73.5$^{bc}$ | 26.5$^{bc}$ |
| RD20 | 78.4$^{ab}$ | 21.6$^{cd}$ |
| RD33 | 70.4$^{cd}$ | 29.6$^{ab}$ |
| LSD (5%) | 6.115 | 6.115 |

1. Values followed by the same letter(s) in a column are not signiûcantly different at 5% level ($P$ <0.05).

## Conclusions

The present study reconfirms the initial finding that the drought responsive transcription factor, *DREB1A* carrying transgenic events of groundnut show induction of root response and signifcant increase in root:shoot ratio (Vadez et al. 2007). However, the previously observed differences in total water extracted as shown by Vadez et al. (2007) were not confirmed. This is explained by the fact that while the previous study was done under terminal drought condition with only one drying cycle, in the present study, an intermittent stress was imposed, whereby plants received water, which may have allowed all the tested genotypes to eventually explore all of the soil profile and extract similar amounts of water. Moreover, the enhanced root:shoot ratio showed a positive influence on pod and seed yield under the imposed intermittent drought stress while TE showed no correlation with the yield. Thus, the enhanced performance of the transgenic plants of groundnut, over-expressing the *DREB1A* transcription factor under the contained field trial supports the higher root:shoot ratio, due to biomass partitioning advantageously more towards roots and pods and lesser towards shoot in the transgenic events in general, which could prove to be an important trait for producing better yield by extracting efficiently the water from deep soil layers.

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