Stomatal clustering pattern in
Arachis hypogaea L. under
water deficit stress

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Soil health, water use, crop yield and biodiversity are directly affected by climate change and drought is one of the most severe consequences of climate change. Accordingly, plants undergo physiological manipulations, such as differential stomatal behaviour which ultimately affect the water use efficiency (WUE), role of various chemicals on adaxial stomatal behaviour, gaseous exchange capacity and yield. Here, we investigated these aspects in groundnut varieties of Spanish bunch type suitable for summer cultivation. Type A clustering of stomata (two stomata in direct contact laterally without intervening epidermal cells between neighbouring guard cells) was recorded accounting 3.3% in well-watered plants which increased due to water deficit (WD) treatments. Foliar application of ethrel and 8-HQ increased clustering of stomata by 45 and 47%, respectively under WD stress. Based on our study, groundnut is now included in group of terrestrial plants which showed type A stomatal clustering.

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Crop yield, water use, soil health and biodiversity are directly affected by climate change which include increased temperature, precipitation and hydrological cycle1. The drought is one of the most significant but severe consequences of climate change affecting the agricultural production2. In India, the annual production of groundnut for 2015-16 was 7.2 million tonnes and for 2016-17 it is estimated at 6.50 million tonnes3,4. Stomatal behaviour differs among plant functional types according to their marginal carbon cost of water use and a global relationship of the stomatal behaviour with climate have been demonstrated recently5. Groundnut production fluctuates considerably as a result of rainfall variability due to its underground fruiting habit and low moisture availability during drought.

As a strategy to overcome the adverse impacts of drought, genotypes with relatively lesser maturity period are preferred in low rainfall area. Such genotypes, by virtue of climate physiology, acquire ‘drought escape’ trait6. Changes in morphology, physiology and molecular aspects have been studied in many crops under water deficit stress which revealed that the drought tolerance mechanism impacts the expression patterns of several genes related to drought tolerance. Recently, micro RNA imparting drought tolerance have been reported in Hevea brasiliensis7. Being the gateway of gaseous exchange, the stomata have a critical role as regulators of two major processes between the plant and the environment; the loss of water through the transpiration and the photosynthesis. Clustering of stomatal in the form of groups of two or more stomata with direct contact have been found only in a few plant species to data with exception in Cinnamomum camphora in which clustering of stomata is a very common phenomenon8. Abscisic acid (ABA) limits initiation of stomatal development and induces enlargement of pavement cells. It upregulates gene expression of SPCH and MUTE genes which are master regulators of formation of stomata9. The metabolic inhibitor phenyl mercuric acetate (PMA) has been shown as the most effective anti-transpirant through a specific effect on the guard cells. The involvement of ethylene in regulating stomatal and photosynthetic responses is not clear as contradictory claims have been made on the effects of ethylene on stomatal opening and photosynthesis10. The metal chelate 8-HQ is an important growth retardant11 and succinic acid is considered as the mimic of salicylic acid12. In groundnut, even though having equal numbers and sizes of stomata on adaxial and abaxial leaf surf, the net photosynthetic rate ($P_n$) is not same on these surfaces13.

Stomatal behaviour under water deficit (WD) stress in combination with foliar application of different chemicals is not clearly reported in groundnut. Therefore, in this study, we tried to manipulate the stomatal behaviour so as to characterize its impact of
gaseous exchange and yield in groundnut under WD stress condition. PMA and HQ are known to be toxic, while ABA and ethrel are too expensive for field application and thus, these chemicals are commercially not viable. However, as a part of the basic research, these chemicals along with succinic acid were selected in this field experiment designed to study their impacts on stomatal behaviour, transpiration and net photosynthetic rate in four groundnut varieties under restricted water supply.

Materials and Methods
A field experiment was conducted during dry season (January-June) in year 2014 at the research farm of ICAR-Directorate of Groundnut Research, Junagadh, Gujarat (lat. 21°31' N, Long 70°36' E) in the Vertic Ustochrept soil with pH of 8.5 and electrical conductivity of 0.16 dSm⁻¹. Most of the groundnut varieties under cultivation are Spanish and Virginia bunch type genotypes having relatively shorter crop duration as compared to the Virginia runner. In this study also, four Spanish bunch type groundnut varieties i.e. ICGV 86031, Kadiri 9, ICGV 91114 and VRI 2 were raised at different water supply viz., the control to replenish the PAN evaporative demand and the water deficit (WD) in combination with different foliar chemical treatments in split plot design. The treatment combinations were T₁: Control (well-watered) in which the quantity of irrigation water supplied to replenish the cumulative PAN evaporation at three days interval; T₂: water deficit (WD) (treated control, 25% of T₁); T₃: T₂ + distilled water spray; T₄: T₂ + ABA @ 50 µM; T₅: T₂ + PMA @ 100 mg/L; T₆: 25% of T₁ + Ethrel @ 1000 mg/L; T₇: T₂ + 8-HQ@10 mg/L and T₈: T₂ + succinic acid @150 mg/L. Foliar chemical application was done twice viz., first at 45 DAS and second at 60 DAS. Observations on stomatal behaviour were recorded as per described by Orcen et al. The occurrence of stomatal clusters in 20 random selected fields counted and the incidence of stomatal clusters under each slide was calculated and expressed as per cent incidence of clustering. Intrinsic water use efficiency (WUEᵢₑ) was calculated as $P_{n}/E$.

Results and Discussion
At 75 days after sowing (DAS) there was almost 50% reduction in soil moisture content (SMC) in all WD treatments as compared to the absolute control and the stomata density was reduced (data not presented) but the occurrence of contiguous clustering of stomata was increased under WD treatments. The percentage incidence of stomatal clustering was only 3.3% in control plants. All WD treatments have increased incidence of the stomatal clustering however, the percentage incidence of stomatal clustering increased to the highest of 47% by foliar ethrel application and to 45% by 8-HQ application. The clustering pattern of stomata was analysed and found that type A (two stomata in direct contact laterally without intervening epidermal cells between neighbouring guard cells) clustering of stomata is reported in groundnut (Fig. 1). About 32% reduction in stomatal conductance ($g_s$) was resulted due to WD treatments and varieties in descending order for their conductance were Kadiri 9 > ICGV 86031 > VRI 2 > ICGV 91114 and varieties in descending order for rate of photosynthesis (data not presented) were

![Fig. 1 — Microscopic view of the adaxial surface of groundnut leaf (A) general view at 10x; (B) stomatal clustering viewed at 20x; (C) at 40x; and (D & E) at 100x. (Inside circles is type A clustering of stomatal in groundnut (Kadiri 9) adaxial leaf surface)](image-url)
Kadiri 9 > ICGV 86031 > ICGV 91114 > VRI2. The intrinsic water use efficiency (WUE$_{int}$) had increased due to water deficit stress. Mean value of WUE$_{int}$ was 3.79 in WD treatments and 2.96 in well-watered plants (data not presented). Though the WUE$_{int}$ was not varied among the varieties, the highest WUE$_{int}$ was in ICGV 91114 in combination with ABA spray.

To optimize dry matter production, stomata function in a way to balance photosynthesis and transpiration. Small sized stomata can open and close more rapidly and thus, the opening and closing mechanism operates at the best which is the most important requirement during maximizing CO$_2$ diffusion besides preventing water loss under limited water availability. Reduced pore size indicates closing of stomata. This is a specific response to diminished water availability. As per the theory of “one cell spacing rule”, stomata are found evenly spaced, and almost never next to each other; there must be at least one cell between two stomata. However, a stomatal cluster is a term for a group of two or more stomata that make direct contact. In our study, decreased stomatal density even against increased incidence of contiguous type A clusters (as described by Gan - two stomata in direct contact laterally without intervening epidermal cells between neighbouring guard cells) (Fig. 1) explains that osmotic stress caused by drought impaired normal stomatal patterning and produced more contiguous stomatal clusters in the leaf epidermis.

Modification of transpiration efficiency under stressful environments is found to be regulated by action of the ERECTA gene, which co-regulates stomatal patterning with ERL1 and ERL2. Stomatal clusters are reported in only in a few plant species to data with exceptions like *Cinnamomum camphora* and *Vicia faba* and a few more. A similar stomatal patterning in the *Arabidopsis thaliana* mutant line four lips, suggested the impaired symmetric divisions of guard mother cells during stomatal differentiation by osmotic stresses. Based on our study, groundnut is now included in group of terrestrial plants which showed type A stomatal clustering.

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