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Foliar application of humic acid and seaweed extract modulates proline accumulation and chlorophyll content in custard apple under drought conditions

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Abstract

Drought stress triggers osmotic adjustment through proline accumulation and compromises photosynthetic potential through chlorophyll degradation in fruit crops. Biostimulants offer sustainable approaches to enhance stress tolerance through coordinated metabolic regulation, though underlying mechanisms remain poorly characterized in underutilized species like custard apple (*Annona squamosa* L.). This study provides the first field assessment of proline content and chlorophyll concentration responses to foliar-applied humic acid (HA) and seaweed extract (SWE) under drought conditions in custard apple. Sixteen treatment combinations comprising HA (1000–3000 ppm) and SWE (1000–3000 ppm), applied individually and in combination, were evaluated across two contrasting drought seasons (2022–2023) using the custard apple cv. Balanagar. Proline content and total chlorophyll were quantified before (June) and after (November–December) biostimulant applications under natural drought stress. Combined HA (3000 ppm) + SWE (2000 ppm) achieved optimal metabolic modulation, reducing proline accumulation by 13.2% (from 9.54 to 8.28 $\mu\text{mol g}^{-1}$ FW) and enhancing chlorophyll retention by 6.9% (from 2.32 to 2.48 mg g^{-1} FW) relative to untreated controls. Individual high-concentration treatments (HA 3000 ppm; SWE 2000 ppm) provided significant but comparatively lower benefits. Principal component analysis revealed distinct treatment-dependent temporal trajectories, with PC1 and PC2 explaining 83% of variance in proline dynamics and coordinated metabolic responses. Year-to-year consistency despite 34% rainfall reduction (1095 vs. 718 mm) demonstrated robust stress mitigation across variable environmental conditions. Observed physiological patterns are consistent with complementary mechanisms involving HA-mediated nutrient and water relations improvements, and SWE-associated hormonal and antioxidant effects, as reported in the literature for other crops; however, these mechanisms were not directly measured in the present study. Strong inverse correlation between proline content and chlorophyll retention ($r = -0.67$ to -0.73 , $P < 0.001$) indicates coordinated regulation of osmotic adjustment and photosynthetic pigment maintenance. These findings support the use of proline and chlorophyll



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as practical physiological indicators for monitoring stress responses and evaluating biostimulant effects in custard apple production systems under water-limited conditions.

Keywords Abiotic stress, Biostimulants, Chlorophyll metabolism, Osmotic adjustment, Proline homeostasis, Stress physiology

1 Introduction

Custard apple (*Annona squamosa* L.), belonging to the family Annonaceae, is an economically important underutilized fruit crop cultivated across tropical and subtropical regions worldwide [25]. Despite its considerable nutritional value, medicinal properties, and market potential, commercial cultivation faces substantial challenges from environmental stress conditions, particularly during critical growth and reproductive phases [39]. Abiotic stress responses in custard apple involve complex metabolic adjustments that balance resource allocation between stress protection and growth maintenance, yet the physiological mechanisms underlying stress tolerance remain poorly characterized compared to model crop species.

1.1 Drought and salinity stress impacts on custard apple physiology

Root-zone hydrothermal conditions strongly influence productivity, with prolonged soil heat and seasonal drought significantly reducing growth, fruit quality, and yield parameters [47]. Optimal root-zone moisture management, maintaining approximately 75% field moisture capacity, produces the highest yields, increasing productivity by 33.99% compared to the lowest-moisture treatments in 2-year field trials conducted on *A. squamosa* [47]. These dramatic yield differences underscore the sensitivity of custard apple to water deficit stress and highlight the need for effective drought mitigation strategies.

Salinity stress represents another major abiotic constraint limiting custard apple production in semi-arid regions. Irrigation water at EC_w 4.0 dS m⁻¹ significantly decreases chlorophyll a, total chlorophyll, and carotenoids while lowering photosystem II quantum efficiency (Fv/Fm) and causing photo-inhibitory damage in *A. squamosa* seedlings [15]. Field studies document that salinity at EC_w 3.0 dS m⁻¹ substantially reduces fruit number, fresh fruit mass, and seed mass in the third cultivation year [39]. Temperature extremes during nursery establishment and extended irrigation intervals further restrict gas exchange parameters and overall physiological activity, though exogenous proline application has demonstrated potential for partial physiological rescue under severe water stress conditions [43]. These findings collectively demonstrate that custard apple exhibits measurable sensitivity to multiple abiotic stressors through disruption of both osmotic homeostasis and photosynthetic function.

1.2 Proline homeostasis as a stress-responsive metabolic marker

Proline accumulation represents a universal response to osmotic challenges, including salinity and water deficit stress, as well as temperature extremes, though accumulation patterns vary considerably by species, tissue type, stress intensity, and duration [41]. The major biosynthetic route proceeds from glutamate via Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR), with tightly regulated catabolism through proline dehydrogenase (ProDH) enabling rapid metabolic turnover upon stress relief [24, 45].

Proline contributes multiple protective functions at cellular and molecular levels: (1) lowering cellular osmotic potential to retain water and sustain turgor pressure during drought or salinity exposure through compatible solute effects, (2) stabilizing membrane structures and protein complexes against ionic, dehydration, or temperature-induced damage through direct molecular interactions, (3) supporting cellular redox balance during both stress imposition and recovery phases through its role in reactive oxygen species (ROS) scavenging, and (4) functioning as a molecular chaperone that prevents protein aggregation and maintains enzyme activities under denaturing conditions [4, 21, 38, 45].

However, elevated proline concentrations exhibit context-dependent physiological significance. High proline can reflect either adaptive osmoprotective responses in stress-tolerant genotypes or symptom-level cellular injury in susceptible lines, making proline quantification most informative within comprehensive multi-metric screening approaches that incorporate complementary measurements of other compatible solutes, antioxidant enzyme activities, and stress-responsive gene expression markers [16, 41]. The magnitude and timing of proline accumulation relative to stress onset, combined with recovery kinetics following stress relief, provide quantitative indicators of stress severity and adaptive capacity that can guide stress management interventions.

1.3 Chlorophyll dynamics and photosynthetic stress responses

Chlorophyll content exhibits tight linkage to photosynthetic capacity and crop productivity across diverse fruit species, with higher leaf chlorophyll concentrations typically supporting greater light capture efficiency and carbon assimilation rates [13, 20]. Drought and salinity stresses reduce stomatal conductance, electron transport chain efficiency, and CO₂ assimilation capacity, frequently accompanied by decreases in leaf chlorophyll concentration and chlorophyll fluorescence parameters such as maximum quantum efficiency of photosystem II (Fv/Fm), collectively explaining substantial reductions in vegetative growth and fruit yield under stress conditions [5, 31].

Chlorophyll fluorescence parameters reveal functional damage to photosystem II reaction centres and electron transport chain components under environmental stress, enabling early detection of photosynthetic impairment before manifestation of significant yield losses [27]. Leaf chlorophyll concentration serves as a practical, non-destructive indicator of plant nutritional status and environmental stress responses, exhibiting sensitive responses to nutrient deficiencies, ionic toxicity, and environmental pollutants [11]. The mechanisms underlying stress-induced chlorophyll degradation involve both reduced biosynthesis through downregulation of chlorophyll biosynthetic genes (CHLH, CHLI, CHLD encoding magnesium chelatase subunits) and accelerated catabolism through upregulation of senescence-associated genes, including non-yellow colouring 1 (NYC1), stay-green (SGR), and pheophorbide a oxygenase (PAO) [50].

1.4 Metabolic integration of proline and chlorophyll responses

Proline and chlorophyll metabolism exhibit complex interdependencies under stress conditions that extend beyond simple independent responses. Proline biosynthesis via the glutamate pathway (P5CS-P5CR) directly competes with chlorophyll synthesis for glutamate precursors, creating metabolic trade-offs between osmoprotection and photosynthetic capacity [48]. Glutamate serves as the common nitrogen donor for both

pathways: in proline biosynthesis, glutamate is phosphorylated and reduced to P5C by P5CS, while in chlorophyll synthesis, glutamate undergoes aminotransferase-mediated conversion to 5-aminolevulinic acid (ALA), the universal precursor for all tetrapyrroles, including chlorophyll [42]. Under severe stress conditions, preferential allocation of glutamate toward proline biosynthesis may occur at the expense of chlorophyll synthesis, particularly when nitrogen availability becomes limiting.

Additionally, proline-mediated ROS scavenging indirectly protects chlorophyll from oxidative degradation by reducing photo-oxidative damage to photosystem reaction centres [38, 41]. Proline reacts directly with hydroxyl radicals ($\cdot\text{OH}$) and singlet oxygen ($^1\text{O}_2$), two particularly damaging ROS species generated in chloroplasts during photosynthetic electron transport under stress conditions. By intercepting these ROS before they can attack chlorophyll molecules or thylakoid membrane lipids, proline accumulation provides antioxidant protection that preserves photosynthetic apparatus integrity. This protective function operates in parallel with enzymatic antioxidant systems (SOD, CAT, APX) to maintain chloroplast redox homeostasis.

The coordinated regulation of these pathways suggests that effective stress mitigation strategies should simultaneously: (1) reduce excessive proline accumulation that indicates severe stress while maintaining sufficient osmoprotection for cellular function, (2) preserve chlorophyll content and photosystem integrity to sustain carbon assimilation and energy generation, and (3) balance nitrogen allocation between stress-protective and growth-promoting metabolic functions to optimize whole-plant resource economics. Biostimulants may influence this metabolic integration through multiple entry points, including hormonal regulation of gene expression (particularly ABA-responsive transcription factors controlling P5CS and senescence-associated genes), antioxidant enzyme enhancement reducing oxidative triggers for both proline synthesis and chlorophyll degradation, and nutrient optimization that alleviates competition for limiting substrates like glutamate. However, specific mechanisms remain incompletely characterized in perennial fruit crops, particularly regarding the temporal dynamics of coordinated metabolic responses and the optimal intervention strategies for field production systems.

1.5 Biostimulants in horticultural stress management

Biostimulants have emerged as sustainable biotechnological tools for enhancing plant stress tolerance and physiological performance in horticultural crop production systems [35]. Humic substances, derived from the chemical and biological humification of plant and animal residues through microbial metabolism, function as multi-target biostimulants that mimic plant hormones, modulate signalling pathways, stimulate plasma-membrane transport systems, and enhance antioxidant enzyme activities, collectively improving root architecture, nutrient availability, stress tolerance, and fruit-crop performance [10].

These bioactive compounds exhibit auxin-like activity that induces lateral root formation through activation of NAC family transcription factors (particularly NAC1 and NAC4) and increases plasma-membrane H^+ -ATPase activity, energizing secondary active transport of ions and enabling acid-growth-type cell expansion in root tissues [10, 19]. Humic acid-induced root architectural modifications typically include increased lateral root density, enhanced root hair formation, and greater overall root biomass

allocation, collectively expanding the soil volume accessible for water and nutrient acquisition—a particularly valuable adaptation under water-limited conditions.

Under abiotic stress conditions, humic acids commonly increase activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) while simultaneously lowering malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) accumulation, thereby protecting cellular membranes from oxidative damage [17, 32]. At the molecular level, humic acids modulate expression of P5CS under salinity stress, altering proline synthesis pathways and osmotic adjustment mechanisms [19]. Transcriptomic studies reveal that humic acid treatments frequently increase expression or accumulation of growth-promoting hormones (indole-3-acetic acid, brassinosteroids) and reduce stress-responsive hormones (abscisic acid, salicylic acid, jasmonic acid), effectively shifting plants from stress-arrested metabolic states toward growth maintenance and stress acclimation rather than acute stress responses [32].

Field and greenhouse studies report significantly increased fruit number, size, soluble solids concentration, and fruit retention following foliar or soil-applied humic acid applications in mango (*Mangifera indica*), tomato (*Solanum lycopersicum*), and other commercially important fruit crops. These productivity improvements reflect the integrated effects of enhanced nutrient acquisition, improved water relations, maintained photosynthetic capacity, and optimized carbon partitioning toward reproductive development.

Seaweed extracts similarly function as multi-component biostimulants containing diverse bioactive compounds including measurable concentrations of auxins (indole-3-acetic acid), cytokinins (zeatin, zeatin riboside), gibberellins, abscisic acid, and defense-related hormones (salicylic acid, jasmonic acid) that provide direct hormone-like signalling to treated plants [12]. Sulfated polysaccharides, including laminarins, ulvans, carrageenans, and alginates, function as pathogen-associated molecular pattern (PAMP)-like elicitors and osmoprotectant, triggering salicylic acid, jasmonic acid, and ethylene-mediated defense pathways in plant tissues while also contributing to cellular osmotic adjustment [9, 12]. Phenolic compounds, flavonoids, and quaternary ammonium compounds such as betaines enhance antioxidant capacity and osmotic adjustment, supporting ROS scavenging and osmoprotection under environmental stress conditions [9, 12].

Recurring soil applications of *Durvillaea potatorum* and *Ascophyllum nodosum* extracts increased wine-grape (*Vitis vinifera*) yield by 14.7% across seven multi-year field trials conducted in diverse viticultural regions of Australia, demonstrating reproducible efficacy under varying environmental conditions [30]. In tomato production systems, foliar or root treatments with brown-seaweed extracts produced significantly larger root and shoot systems, accelerated flowering phenology, and increased individual fruit weight, reducing salinity-related fruit number loss from approximately 28.7–3.4% compared to untreated controls [49].

Seaweed treatments improve fruit biochemical composition through increased soluble solids, reducing and total sugars, and anthocyanins in strawberries (*Fragaria × ananassa*) and tomatoes, and elevated polyphenol content and antioxidant activity in blueberry (*Vaccinium* spp.), attributed to hormonal modulation of secondary metabolism, enhanced nutrient uptake capacity, and activation of phenylpropanoid biosynthetic

pathways [40, 44, 49]. At the metabolic level, cytokinin-rich seaweed extracts delay leaf senescence and maintain chlorophyll content under stress conditions by downregulating senescence-associated genes (SAGs) and chlorophyll catabolic enzymes, preserving photosynthetic capacity during critical reproductive growth phases.

Biostimulant applications frequently increase leaf chlorophyll concentration, net photosynthetic rates, and reproductive yield components in commercial fruit crop production. Controlled studies in apple (*Malus domestica*), olive (*Olea europaea*), and strawberry demonstrate that foliar applications of moringa (*Moringa oleifera*) leaf extract, seaweed extracts, fulvic acid, chitosan, and silicon significantly increase chlorophyll content, vegetative growth parameters, fruit set percentage, and marketable yield under both optimal and environmentally stressful growing conditions [1, 23, 35]. The mechanistic basis for these improvements involves multifactorial processes, including enhanced nitrogen uptake and assimilation supporting chlorophyll biosynthesis, antioxidant protection against chlorophyll degradation, and cytokinin-mediated suppression of senescence pathways.

1.6 Research gaps in *Annona* biostimulant applications

Despite documented benefits in numerous fruit crop species, research on biostimulant applications specifically in *Annona* species remains substantially limited, with significant gaps in mechanistic understanding. A field randomized block study demonstrated that humic substance applications increased fruit soluble-solids content and pH while producing a yield enhancement of approximately 0.63 t ha⁻¹ when combined with 100 g nitrogen per plant in custard apple cultivation [14]. A commercial orchard trial showed that humic substance application (Ks100 product) increased leaf potassium content, though effects on other mineral nutrients were inconsistent, and no significant changes in soil chemical attributes were observed during the experimental period [6].

However, published *Annona* field trials have predominantly measured agronomic yield and post-harvest fruit-quality parameters while neglecting mechanistic physiological markers such as leaf gas exchange characteristics, compatible osmolytes (proline, glycine betaine), antioxidant enzyme activities (SOD, CAT, APX, POD), or root architectural responses, nor have these studies comprehensively quantified plant responses to imposed abiotic stress conditions with appropriate stress intensity characterization [6, 14]. This represents a critical knowledge gap, as understanding the physiological mechanisms through which biostimulants enhance stress tolerance is essential for optimizing application protocols, predicting treatment efficacy under varying environmental conditions, and developing cultivar-specific recommendations.

Critically, no studies have integrated simultaneous measurements of proline homeostasis and chlorophyll dynamics with biostimulant applications in custard apples to evaluate the stress mitigation potential and elucidate the underlying physiological mechanisms. Given the metabolic interdependencies between these pathways and their central roles in stress response and photosynthetic function, coordinated measurement of both parameters provides mechanistic insights into how biostimulants modulate resource allocation and metabolic prioritization under stress. The majority of research targets *A. squamosa*, while other commercially cultivated *Annona* species, including *A. atemoya*, *A. cherimola*, *A. muricata*, and *A. reticulata* lack controlled biostimulant evaluation trials [14, 25].

Existing trials exhibit considerable variation in commercial product formulation, seaweed source species, application concentration, timing protocols, and environmental conditions, with standardized evidence-based protocols lacking for practical implementation in commercial production systems [6, 14, 37]. This methodological heterogeneity complicates meta-analysis and synthesis of results across studies. Furthermore, no *Annona* studies have investigated biostimulant treatment effects on stress-responsive gene expression profiles (P5CS, ProDH, ABA biosynthesis genes, antioxidant enzyme genes) or intracellular signalling pathway activation (ABA, ROS, calcium signalling cascades), substantially limiting mechanistic understanding of modes of action and cultivar-specific variability in treatment responses [25]. The absence of molecular-level investigations represents a significant barrier to the rational development and optimization of biostimulants for this economically important but scientifically underexplored fruit crop.

1.7 Research objectives and hypotheses

Based on documented biostimulant effects in annual crops and the mechanistic framework of stress-responsive metabolism outlined above, we hypothesized that: (1) foliar application of humic acid and seaweed extract would reduce drought-induced proline accumulation through improved plant water status (enhanced root water uptake, reduced transpirational water loss) and reduced oxidative stress burden (enhanced antioxidant enzyme activities lowering ROS-mediated stress signalling), (2) biostimulant treatments would enhance chlorophyll retention through improved nitrogen nutrition supporting biosynthesis and cytokinin-mediated delay of senescence-associated chlorophyll catabolism, and (3) synergistic combinations would provide superior metabolic benefits compared to individual applications through complementary modes of action—humic acid primarily operating through root-zone effects on water and nutrient acquisition, while seaweed extract provides foliar-absorbed hormones and metabolites directly influencing leaf metabolism.

Specific objectives were to: (1) quantify concentration-dependent effects of humic acid (HA, 1000–3000 ppm) and seaweed extract (SWE, 1000–3000 ppm) on proline dynamics under natural field drought conditions across two contrasting rainfall years, (2) assess chlorophyll homeostasis responses to biostimulant applications across critical reproductive growth stages (flowering through fruit maturation), (3) identify optimal concentration combinations for synergistic stress mitigation through factorial evaluation of HA $\times$ SWE interactions, (4) establish temporal patterns of metabolic adjustment using multivariate analysis (principal component analysis) to visualize treatment clustering and identify key response trajectories, and (5) develop mechanistic interpretations linking observed biostimulant-induced metabolic changes to stress tolerance mechanisms documented in model systems, providing a physiological framework for understanding biostimulant efficacy in custard apple production.

This research provides foundational physiological data to support the integration of biostimulants into environmentally sustainable custard apple production systems and establishes proline content and chlorophyll concentration as practical biomarkers for monitoring stress intensity and evaluating stress management interventions in commercial orchards.

2 Materials and methods

2.1 Plant material and experimental site

The field experiment was conducted during 2022 and 2023 at the research farm of College of Horticulture and Forestry, Jhalawar (24.5939° N, 76.1643° E, 261 m above sea level), Rajasthan, India. The experimental site experiences a semi-arid tropical climate with hot summers and mild winters. Twelve-year-old custard apple (*Annona squamosa* L.) cv. Balanagar trees maintained under commercial orchard management practices were used. Trees were spaced at 5 × 5 m and trained to an open centre system.

2.2 Plant material identification and voucher specimen

The custard apple cultivar 'Balanagar' (*Annona squamosa* L., Annonaceae) is a commercially cultivated selection originally sourced from the Regional Horticultural Research Station, Vengurla, Maharashtra, India, and established at the experimental farm in 2010. Dr. Jitendra Singh, Department of Fruit Science, College of Horticulture and Forestry, Jhalawar, identified the plant material. A voucher specimen (reference number: CHF/JLW/AS/2022/01) was deposited at the institutional herbarium of the College of Horticulture and Forestry, Jhalawar (24.5939° N, 76.1643° E), Rajasthan, India.

The soil was classified as Vertisols with pH 7.85, electrical conductivity 0.58 dS m⁻¹, organic carbon 0.46%, and available nitrogen, phosphorus, and potassium levels of 286.56, 15.21, and 210.00 kg ha⁻¹, respectively. During 2022, total rainfall was 1095 mm (July–September), while 2023 received 718.30 mm, indicating substantially lower precipitation and more severe drought stress. Maximum temperatures ranged from 40 to 46 °C (April–June) and 22.50–23.20 °C (December–February). Minimum temperatures ranged from 20.10 to 21.40 °C in summer and 9.00–10.20 °C in winter. The mean relative humidity varied from 37.70% during the summer to 82.00% during the monsoon.

Meteorological data were recorded from an automated weather station located 500 m from the experimental plot. Soil volumetric water content was monitored at 0–30 cm depth using time-domain reflectometry (TDR sensor Model TDR 310H) in three representative plots per treatment during key growth stages.

2.3 Experimental design and treatments

The experiment was laid out in a factorial randomized block design with three replications, comprising 16 treatment combinations investigating individual and interactive effects of foliar-applied humic acid (HA) and seaweed extract (SWE). The experimental design consisted of 48 trees (4 rows × 12 trees per row) covering a total plot area of 60 m × 15 m. Treatment structure consisted of:

- Control (T1): Distilled water spray
- Individual HA treatments (T2–T4): 1000, 2000, and 3000 ppm
- Individual SWE treatments (T5–T7): 1000, 2000, and 3000 ppm
- Combined HA × SWE treatments (T8–T16): Nine combinations of HA (1000, 2000, 3000 ppm) and SWE (1000, 2000, 3000 ppm)

Humic acid (IFFCO Humifco Bio-stimulant, containing humic acid, fulvic acid, and bio potash) and seaweed extract (Sagarika, IFFCO, Delhi; derived from *Kappaphycus alvarezii* and *Sargassum* species harvested from Southern coasts of India, containing 28% w/w essential proteins, carbohydrates, vitamins, minerals, inorganic salts, plant growth

hormones, and bioactive substances including betain and mannitol) were measured at 1, 2, and 3 mL L⁻¹ water to achieve respective treatment concentrations.

2.4 Cultural practices and biostimulant application

Standard cultural practices were uniformly maintained across all plots. Irrigation was provided weekly during summer (March–June) through drip irrigation, utilizing two drippers per tree with a combined discharge capacity of 16 L per plant per irrigation event. No supplemental irrigation was applied during monsoon (July–September) due to natural rainfall. Supplemental drip irrigation resumed in October until December. Canopy area was maintained weed-free through manual weeding at 15-day intervals.

Uniform fertilizer application was provided at 400 g N, 250 g P₂O₅, and 400 g K₂O per tree per year, split into two applications during July and August. Integrated pest and disease management practices were implemented uniformly following standard recommendations.

Foliar applications were conducted using a hand-operated knapsack sprayer calibrated for uniform canopy coverage during early morning hours (0600–0800 h) under calm wind conditions. Two foliar spray applications were implemented during each year: first spray during the first week of July (coinciding with flowering initiation) and second spray during the first week of August (coinciding with fruit set stage). Treatment solutions were freshly prepared immediately before each application. Control trees received equivalent volumes of distilled water spray. A non-ionic surfactant (Tween-20) was added at 0.05% v/v to all treatment solutions. Approximately 3 L of spray solution were applied per tree to achieve thorough canopy coverage without runoff.

2.5 Proline content determination

Leaf samples for proline quantification were collected from fully expanded, mature leaves (90–120 days post leaf emergence) from the middle third of the current season's growth during early morning hours (0600–0800 h) to minimize diurnal variation. Samples were collected in two phases: initial sampling in June before treatment application (pre-treatment baseline) and final sampling in November–December (post-treatment evaluation). Samples were immediately placed in ice-cooled containers and transported to the laboratory.

Proline content was measured following the ninhydrin colorimetric method [8]. Fresh leaf tissue (5 g) was homogenized in 10 mL of 3% (w/v) sulfosalicylic acid using a pre-chilled mortar and pestle. The homogenate was filtered through Whatman No. 2 filter paper. To 2 mL of the clear supernatant, 2 mL of acid ninhydrin reagent (containing 1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 60 min, then rapidly cooled in an ice bath. Four millilitres of toluene was added and vigorously vortexed for 15 s. The chromophore-containing toluene layer was separated and the absorbance was measured at 520 nm using a UV–visible spectrophotometer against a toluene blank.

The method was validated using L-proline standards (Sigma-Aldrich, purity ≥ 99%). Calibration curves demonstrated linearity ($R^2 > 0.998$) across the concentration range 0–80 µg mL⁻¹. Recovery efficiency from spiked leaf extracts was 96.3 ± 2.1% (mean ± SD, n = 6). Intra-assay coefficient of variation was 2.8% and inter-assay CV was 4.3%. The

detection limit was $0.5 \mu\text{mol g}^{-1}$ fresh weight. Proline content was calculated using the formula:

$$\text{Proline content } (\mu\text{mol g}^{-1} \text{ fresh weight}) = (\mu\text{g proline mL}^{-1} \times \text{mL toluene} \times 5) / (115.5 \times \text{g fresh weight sample})$$

2.6 Chlorophyll content determination

Total chlorophyll content was determined following the acetone extraction method [36]. Fresh, fully expanded leaves (1 g) from the middle canopy were finely cut and ground in a pre-chilled mortar with 20 mL of 80% (v/v) acetone and acid-washed sand. The homogenate was centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was collected in a 100 mL volumetric flask. Extraction was repeated with fresh aliquots of 80% acetone until the residue became colourless. The combined extracts were made up to 100 mL with 80% acetone. Absorbance was measured at 645 nm and 663 nm using a UV–visible spectrophotometer against an 80% acetone blank.

The method showed extraction efficiency exceeding 98% with an intra-assay CV of 1.9% and an inter-assay CV of 3.2%. Spectrophotometric determinations were performed using quartz cuvettes with 1 cm path length, with all readings conducted within 2 h of extraction. Chlorophyll a and chlorophyll b were determined individually using equations of Arnon [3]:

$$\text{Total Chlorophyll } (\text{mg g}^{-1}) = [20.2 (A_{645}) + 8.02 (A_{663})] \times V / (1000 \times W)$$

where A_{645} and A_{663} represent absorbance readings at 645 nm and 663 nm, respectively, V is the final volume of extract (mL), and W is the fresh weight of the leaf sample (g).

2.7 Statistical analysis

Data were subjected to analysis of variance (ANOVA) for factorial randomized block designs following Panse and Sukhatme [34]. Treatment effects were evaluated using an F-test at 5% significance level ($\alpha=0.05$). Means were compared using critical difference (CD) values calculated at 5% probability level. Standard error of mean (SEm $\pm$) was calculated for all parameters. Pooled analysis across both experimental years was conducted to evaluate treatment consistency and year $\times$ treatment interactions.

All physiological measurements were performed using three independent leaf samples per tree (technical replicates), with values averaged per tree before statistical analysis. Thus, $n=3$ biological replicates (trees) per treatment per year were used as the unit of analysis in ANOVA. Nine technical replicates per treatment per year were collected but averaged to tree-level means prior to all inferential statistics. Outliers were identified using Grubbs' test ($\alpha=0.05$). Fresh weight measurements were standardized to ± 0.01 g precision. All spectrophotometric measurements included blank corrections.

Principal component analysis (PCA) was performed to identify relationships among treatments, experimental years, and physiological variables. Pearson correlation analysis was conducted to examine relationships between proline content and chlorophyll content across treatments and experimental years.

3 Results

3.1 Year-dependent baseline stress conditions

Initial proline concentrations measured in June before treatment application averaged 14.17 $\mu\text{mol g}^{-1}$ in 2022 and 15.20 $\mu\text{mol g}^{-1}$ in 2023, representing a 7.3% increase that corresponded with more severe baseline drought stress during the second experimental year (pooled analysis across treatments). This elevation correlated directly with reduced cumulative pre-monsoon rainfall of 142 mm in 2023 versus 231 mm in 2022, a 38.5% reduction in early-season precipitation. Maximum pre-monsoon temperatures were also elevated in 2023 (average daily maximum 43.2 °C) compared to 2022 (41.7 °C), intensifying evapotranspiration demand and soil moisture depletion. Initial chlorophyll content showed minimal year-to-year variation (2.48 vs. 2.49 mg g^{-1}), indicating that early-season drought primarily triggered osmotic adjustment responses through proline accumulation rather than immediate photosynthetic apparatus degradation. Non-significant differences in initial proline and chlorophyll values across treatment groups within each year ($P>0.05$) confirmed successful randomization and uniform pre-treatment physiological status. These contrasting seasonal conditions provided an opportunity to evaluate biostimulant efficacy under moderate (2022) versus severe (2023) drought stress scenarios.

3.2 Effects of humic acid on proline accumulation

Individual foliar application of humic acid exhibited concentration-dependent modulation of proline content across both experimental years (Table 1). Pre-treatment proline concentrations ranged from 13.12 to 13.24 $\mu\text{mol g}^{-1}$ in 2022 and 15.13 to 15.41 $\mu\text{mol g}^{-1}$ in 2023, with 2023 demonstrating approximately 15% higher initial values corresponding to more severe drought stress associated with reduced total rainfall (718.30 mm vs. 1095 mm).

Following humic acid applications, all treatments demonstrated significant reductions in final proline content relative to the control, with stress mitigation efficiency increasing progressively with concentration. In 2022, final proline content declined from 8.46 $\mu\text{mol g}^{-1}$ in control (T1) to 8.08, 8.06, and 7.93 $\mu\text{mol g}^{-1}$ in treatments with 1000, 2000, and 3000 ppm humic acid (T2, T3, T4), respectively, with all treatments showing statistically significant differences from control (CD 5% = 0.18). Year 2023 exhibited a parallel trend, with final proline values of 9.58 $\mu\text{mol g}^{-1}$ in control reduced to 9.34, 9.23, and 9.21 $\mu\text{mol g}^{-1}$ in T2, T3, and T4, respectively (CD 5% = 0.22), demonstrating consistent stress amelioration despite higher baseline stress intensity.

Table 1 Individual influence of foliar application of humic acid on proline content ($\mu\text{mol g}^{-1}$) in custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	13.12	8.46	15.21	9.58	14.17	9.02
T2—HA (1000 ppm)	13.14	8.08	15.13	9.34	14.13	8.71
T3—HA (2000 ppm)	13.15	8.06	15.15	9.23	14.15	8.65
T4—HA (3000 ppm)	13.24	7.93	15.41	9.21	14.33	8.57
SEm ($\pm$)	0.19	0.06	0.21	0.08	0.14	0.05
CD 5%	NS	0.18	NS	0.22	NS	0.14

NS not significant

Pooled data analysis substantiated these findings, with pooled initial proline content showing non-significant variation ($14.17\text{--}14.33\ \mu\text{mol g}^{-1}$) across treatments, confirming uniform pre-treatment stress conditions. Pooled final values demonstrated pronounced reductions from $9.02\ \mu\text{mol g}^{-1}$ (control) to 8.71, 8.65, and $8.57\ \mu\text{mol g}^{-1}$ in T2, T3, and T4, respectively ($\text{CD } 5\% = 0.14$). The highest humic acid concentration (3000 ppm) consistently provided superior stress mitigation, achieving 6.3% and 3.9% reductions in final proline content compared to the control in 2022 and 2023, respectively. In pooled analysis, T4 treatment resulted in minimum proline content ($8.57\ \mu\text{mol g}^{-1}$), which was statistically similar to T3 ($8.65\ \mu\text{mol g}^{-1}$) and T2 ($8.71\ \mu\text{mol g}^{-1}$), with all three humic acid treatments exhibiting significantly lower proline concentrations compared to control ($9.02\ \mu\text{mol g}^{-1}$).

3.3 Effects of seaweed extract on proline accumulation

Individual foliar application of seaweed extract demonstrated dose-dependent reduction in proline content across both experimental years (Table 2). Initial proline concentrations were substantially elevated, ranging from 13.06 to $13.29\ \mu\text{mol g}^{-1}$ in 2022 and 14.97 to $15.46\ \mu\text{mol g}^{-1}$ in 2023, reflecting baseline water stress conditions during early growth phase. Following biostimulant applications, all seaweed extract treatments exhibited significant reductions in final proline content compared to the control, with the magnitude of reduction positively correlated with concentration.

In 2022, final proline content decreased from $8.43\ \mu\text{mol g}^{-1}$ in control to 8.12, 8.03, and $7.96\ \mu\text{mol g}^{-1}$ in treatments with 1000, 2000, and 3000 ppm seaweed extract (T2, T3, T4), respectively, with T3 and T4 showing statistically significant differences ($\text{CD } 5\% = 0.18$). Year 2023 displayed similar trends, with final proline values of $9.71\ \mu\text{mol g}^{-1}$ (control) reduced to 9.33, 9.24, and $9.07\ \mu\text{mol g}^{-1}$ in T2, T3, and T4, respectively ($\text{CD } 5\% = 0.22$).

Pooled data analysis confirmed these observations, with pooled initial proline content of $14.37\ \mu\text{mol g}^{-1}$ (control) marginally reduced to 14.22, 14.17, and $14.02\ \mu\text{mol g}^{-1}$ in seaweed extract treatments. Pooled final values showed more pronounced reductions from $9.07\ \mu\text{mol g}^{-1}$ (control) to 8.72, 8.64, and $8.52\ \mu\text{mol g}^{-1}$ in T2, T3, and T4, respectively ($\text{CD } 5\% = 0.14$). The highest seaweed extract concentration (3000 ppm) consistently demonstrated the most effective stress mitigation, achieving 5.6% and 6.6% reduction in final proline content compared to the control in 2022 and 2023, respectively. In pooled analysis, minimum proline accumulation ($8.52\ \mu\text{mol g}^{-1}$) was recorded in T4 treatment (3000 ppm), showing statistical equivalence with T3 treatment ($8.64\ \mu\text{mol g}^{-1}$) but significantly lower than T2 and control treatments.

Table 2 Individual influence of foliar application of seaweed extract on proline content ($\mu\text{mol g}^{-1}$) in custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	13.29	8.43	15.46	9.71	14.37	9.07
T2—SWE (1000 ppm)	13.13	8.12	15.30	9.33	14.22	8.72
T3—SWE (2000 ppm)	13.17	8.03	15.17	9.24	14.17	8.64
T4—SWE (3000 ppm)	13.06	7.96	14.97	9.07	14.02	8.52
SEm ($\pm$)	0.19	0.06	0.21	0.08	0.14	0.05
CD 5%	NS	0.18	NS	0.22	NS	0.14

NS not significant

3.4 Interactive effects of humic acid and seaweed extract on proline accumulation

Combined foliar application of humic acid and seaweed extract revealed significant interactive effects on proline metabolism (Table 3). Initial proline content across treatments ranged from 13.61 to 14.90 $\mu\text{mol g}^{-1}$ (pooled data) with no significant differences among treatments, confirming uniform pre-treatment stress conditions. However, final proline content showed significant interaction effects between humic acid and seaweed extract ($\text{CD}=0.29 \mu\text{mol g}^{-1}$ at 5% level in pooled data), ranging from 8.28 to 9.54 $\mu\text{mol g}^{-1}$.

In 2022, initial proline content before foliar application ranged from 12.94 to 13.36 $\mu\text{mol g}^{-1}$ with non-significant differences. Final proline assessment in December revealed minimum accumulation (7.81 $\mu\text{mol g}^{-1}$) in T15 treatment (humic acid 3000 ppm plus seaweed extract 2000 ppm). This treatment obtained statistical equivalence with T16, T14, T13, T12, T11, T10, T9, T8, T7, T6, and T4 treatments but was significantly lower than T5, T3, T2, and T1 treatments ($\text{CD } 5\% = 0.36$). Maximum proline content (9.24 $\mu\text{mol g}^{-1}$) was recorded in control treatment.

In 2023, initial proline values ranged from 14.27 to 16.40 $\mu\text{mol g}^{-1}$ without significant variation. Final estimation revealed minimum proline accumulation (8.76 $\mu\text{mol g}^{-1}$) in T15 treatment, exhibiting statistical parity with T16, T14, and T12 treatments but significantly lower than remaining treatments ($\text{CD } 5\% = 0.44$). Maximum proline accumulation (9.84 $\mu\text{mol g}^{-1}$) was estimated in control treatment, which was comparable to T2, T3, T5, T7, T9, and T10 but significantly higher than other treatments.

Pooled analysis across both years demonstrated that minimum proline content (8.28 $\mu\text{mol g}^{-1}$) was achieved with T15 treatment (humic acid 3000 ppm plus seaweed extract 2000 ppm), statistically equivalent to T16, T14, T12, and T8 treatments. However, T15 exhibited significantly lower proline accumulation compared to T13, T11, T10, T9, T7, T6, T5, T4, T3, T2, and T1 treatments ($\text{CD } 5\% = 0.29$). Maximum proline

Table 3 Interaction impact of foliar application of humic acid and seaweed extract on proline content ($\mu\text{mol g}^{-1}$) of custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	13.27	9.24	14.94	9.84	14.11	9.54
T2—HA (1000 ppm)	12.98	8.25	15.31	9.61	14.14	8.93
T3—HA (2000 ppm)	13.05	8.20	15.05	9.53	14.05	8.87
T4—HA (3000 ppm)	13.21	8.15	15.54	9.33	14.38	8.74
T5—SWE (1000 ppm)	13.19	8.19	15.19	9.52	14.19	8.85
T6—SWE (2000 ppm)	13.14	8.16	14.81	9.17	13.98	8.67
T7—SWE (3000 ppm)	13.16	8.12	15.16	9.45	14.16	8.78
T8—HA (1000 ppm) + SWE (1000 ppm)	13.01	7.87	15.35	9.20	14.18	8.54
T9—HA (1000 ppm) + SWE (2000 ppm)	13.29	8.14	15.29	9.45	14.29	8.79
T10—HA (1000 ppm) + SWE (3000 ppm)	13.26	8.10	15.60	9.44	14.43	8.77
T11—HA (2000 ppm) + SWE (1000 ppm)	13.10	8.00	15.44	9.24	14.27	8.62
T12—HA (2000 ppm) + SWE (2000 ppm)	12.94	8.00	14.27	8.81	13.61	8.40
T13—HA (2000 ppm) + SWE (3000 ppm)	13.40	8.15	16.40	10.03	14.90	9.09
T14—HA (3000 ppm) + SWE (1000 ppm)	13.15	7.95	15.48	9.09	14.31	8.52
T15—HA (3000 ppm) + SWE (2000 ppm)	13.36	7.81	15.03	8.76	14.20	8.28
T16—HA (3000 ppm) + SWE (3000 ppm)	13.06	7.82	14.73	8.95	13.90	8.39
SEm ($\pm$)	0.38	0.12	0.41	0.15	0.29	0.10
CD 5% (HA $\times$ SWE)	NS	0.36	NS	0.44	NS	0.29

NS not significant

accumulation ($9.54 \mu\text{mol g}^{-1}$) occurred in the control treatment, significantly exceeding all other treatments. A consistent decline in proline content from initial to final measurements was observed across all treatments in both years, with reductions ranging from 3.92 to $5.55 \mu\text{mol g}^{-1}$. Among combined treatments, T13 (humic acid 2000 ppm plus seaweed extract 3000 ppm) exhibited the highest pooled initial proline content ($14.90 \mu\text{mol g}^{-1}$) but demonstrated effective stress modulation with final proline content of $9.09 \mu\text{mol g}^{-1}$.

3.5 Effects of humic acid on chlorophyll content

Individual foliar application of humic acid at different concentrations showed concentration-dependent effects on total chlorophyll content (Table 4). Initial chlorophyll concentrations showed minimal variation across treatments, ranging from 2.46 to 2.50 mg g^{-1} (pooled data), with no significant differences observed between treatments in either year, confirming uniform baseline photosynthetic status.

Final chlorophyll content demonstrated concentration-dependent response to humic acid application, with pooled values ranging from 2.39 to 2.44 mg g^{-1} . In 2022, final chlorophyll assessment in November revealed maximum content (2.44 mg g^{-1}) in T4 treatment (3000 ppm), which was statistically similar to T3 treatment (2000 ppm) but significantly superior to T2 and control treatments ($\text{CD } 5\% = 0.02$). All humic acid treatments exhibited considerably higher chlorophyll than control plants (2.38 mg g^{-1}). During 2023, maximum chlorophyll content (2.44 mg g^{-1}) was again attained in T4 treatment, demonstrating statistical parity with T3 treatment (2.43 mg g^{-1}) but elevated chlorophyll compared to T2 and control treatments ($\text{CD } 5\% = 0.01$). Minimum chlorophyll content (2.40 mg g^{-1}) was recorded in the control treatment.

Pooled analysis across both years revealed that control treatment recorded the lowest pooled final chlorophyll content (2.39 mg g^{-1}), while humic acid at 3000 ppm (T4) exhibited the highest (2.44 mg g^{-1}), representing an improvement of 2.09% over control. Statistical analysis revealed significant differences among treatments for final chlorophyll content ($\text{CD} = 0.01 \text{ mg g}^{-1}$ at 5% level). Maximum chlorophyll content was obtained in T4 treatment, demonstrating statistical equivalence with T3 treatment (2.43 mg g^{-1}) but outperforming T2 and control treatments. Progressive increase in final chlorophyll content was observed with increasing humic acid concentrations, with T2 (1000 ppm), T3 (2000 ppm), and T4 (3000 ppm) recording pooled final values of 2.41, 2.43, and 2.44 mg g^{-1} , respectively.

Table 4 Individual impact of foliar application of humic acid on total chlorophyll content (mg g^{-1}) of custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	2.47	2.38	2.49	2.40	2.48	2.39
T2—HA (1000 ppm)	2.46	2.40	2.49	2.42	2.46	2.41
T3—HA (2000 ppm)	2.47	2.42	2.50	2.43	2.49	2.43
T4—HA (3000 ppm)	2.48	2.44	2.51	2.44	2.50	2.44
SEm ($\pm$)	0.01	0.008	0.01	0.005	0.01	0.005
CD 5%	NS	0.02	NS	0.01	NS	0.01

NS not significant

3.6 Effects of seaweed extract on chlorophyll content

Initial chlorophyll content measured in July before treatment application showed non-significant differences across treatments in both experimental years (Table 5). Pooled initial chlorophyll concentrations ranged from 2.48 to 2.49 mg g⁻¹ with no statistically significant variation, confirming uniform pre-treatment photosynthetic capacity.

Final chlorophyll assessment demonstrated significant treatment effects of seaweed extract application. In 2022, final chlorophyll content ranged from 2.37 to 2.44 mg g⁻¹, with T3 (2000 ppm) recording the maximum value (2.44 mg g⁻¹), significantly superior to the control (2.37 mg g⁻¹) and other seaweed extract concentrations (CD 5% = 0.02). Similarly, in 2023, T3 treatment achieved maximum chlorophyll content (2.45 mg g⁻¹), significantly higher than T4 (2.42 mg g⁻¹), T2 (2.41 mg g⁻¹), and control (2.41 mg g⁻¹) treatments (CD 5% = 0.01).

Pooled analysis demonstrated maximum chlorophyll accumulation (2.45 mg g⁻¹) in T3 treatment (2000 ppm), exhibiting significant superiority over T4, T2, and control treatments (CD 5% = 0.01). Minimum chlorophyll (2.39 mg g⁻¹) was obtained in the control treatment, significantly lower than all seaweed extract treatments. These results indicate that foliar application of seaweed extract at 2000 ppm concentration proved most effective in maintaining elevated chlorophyll concentrations, with a higher concentration (3000 ppm) showing slightly diminished efficacy, suggesting an optimal threshold for seaweed extract-mediated chlorophyll enhancement.

3.7 Interactive effects of humic acid and seaweed extract on chlorophyll content

Combined application of humic acid and seaweed extract demonstrated significant interactive effects on chlorophyll content across both experimental years (Table 6). Initial chlorophyll assessment revealed non-significant differences among treatments in both years, with pooled initial values ranging from 2.46 to 2.52 mg g⁻¹, confirming uniform baseline photosynthetic status.

In 2022, post-harvest analysis demonstrated maximum chlorophyll content (2.49 mg g⁻¹) in T15 treatment (humic acid 3000 ppm plus seaweed extract 2000 ppm), showing statistical parity with T14, T12, T11, and T8 treatments. However, T15 revealed significantly superior chlorophyll accumulation compared to T16, T13, T10, T9, T7, T6, T5, T4, T3, T2, and control treatments (CD 5% = 0.05). Minimum chlorophyll content (2.29 mg g⁻¹) was recorded in the control treatment. In 2023, post-harvest chlorophyll determination revealed maximum content (2.48 mg g⁻¹) in T15 treatment, which was on par with T12 treatment (2.46 mg g⁻¹). T15 demonstrated statistical superiority over

Table 5 Individual effect of foliar application of seaweed extract on total chlorophyll content (mg g⁻¹) in custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	2.47	2.37	2.50	2.41	2.48	2.39
T2—SWE (1000 ppm)	2.48	2.41	2.50	2.41	2.49	2.41
T3—SWE (2000 ppm)	2.48	2.44	2.51	2.45	2.49	2.45
T4—SWE (3000 ppm)	2.47	2.43	2.48	2.42	2.48	2.42
SEm (±)	0.01	0.008	0.01	0.005	0.01	0.005
CD 5%	NS	0.02	NS	0.01	NS	0.01

NS not significant

Table 6 Combined influence of foliar application of humic acid and seaweed extract on total chlorophyll content (mg g^{-1}) of custard apple cv. Balanagar

Treatments	Year I initial (2022)	Year I final (2022)	Year II initial (2023)	Year II final (2023)	Pooled initial	Pooled final
T1—Control	2.50	2.29	2.49	2.35	2.49	2.32
T2—HA (1000 ppm)	2.48	2.38	2.48	2.42	2.48	2.40
T3—HA (2000 ppm)	2.45	2.40	2.52	2.45	2.49	2.43
T4—HA (3000 ppm)	2.45	2.43	2.46	2.39	2.46	2.41
T5—SWE (1000 ppm)	2.46	2.36	2.48	2.43	2.47	2.40
T6—SWE (2000 ppm)	2.45	2.41	2.54	2.41	2.49	2.41
T7—SWE (3000 ppm)	2.44	2.40	2.48	2.44	2.46	2.42
T8—HA (1000 ppm) + SWE (1000 ppm)	2.50	2.44	2.47	2.38	2.48	2.41
T9—HA (1000 ppm) + SWE (2000 ppm)	2.43	2.40	2.51	2.43	2.47	2.42
T10—HA (1000 ppm) + SWE (3000 ppm)	2.47	2.39	2.47	2.39	2.47	2.39
T11—HA (2000 ppm) + SWE (1000 ppm)	2.51	2.47	2.51	2.44	2.51	2.45
T12—HA (2000 ppm) + SWE (2000 ppm)	2.47	2.44	2.53	2.46	2.50	2.45
T13—HA (2000 ppm) + SWE (3000 ppm)	2.48	2.41	2.51	2.42	2.50	2.42
T14—HA (3000 ppm) + SWE (1000 ppm)	2.50	2.46	2.51	2.44	2.51	2.45
T15—HA (3000 ppm) + SWE (2000 ppm)	2.51	2.49	2.53	2.48	2.52	2.48
T16—HA (3000 ppm) + SWE (3000 ppm)	2.45	2.42	2.49	2.43	2.47	2.43
SEm ($\pm$)	0.02	0.02	0.02	0.01	0.02	0.01
CD 5% (HA $\times$ SWE)	NS	0.05	NS	0.03	NS	0.03

NS not significant

T16, T14, T13, T11, T10, T9, T8, T7, T6, T5, T4, T3, T2, and control treatments (CD 5% = 0.03). Minimum chlorophyll (2.35 mg g^{-1}) was determined in the control treatment.

Pooled analysis across both years revealed maximum chlorophyll value (2.48 mg g^{-1}) in T15 treatment (humic acid 3000 ppm plus seaweed extract 2000 ppm), demonstrating statistical equivalence with T14 (2.45 mg g^{-1}), T12 (2.45 mg g^{-1}), and T11 (2.45 mg g^{-1}) treatments. However, T15 application significantly elevated chlorophyll content over T16, T13, T10, T9, T8, T7, T6, T5, T4, T3, T2, and control treatments (CD 5% = 0.03). Minimum chlorophyll status (2.32 mg g^{-1}) was recorded in the control treatment, significantly lower than all other treatments. The T15 treatment combination achieved 6.9% enhancement in chlorophyll content compared to the control, demonstrating the superior efficacy of combined humic acid and seaweed extract application (noting that confirmation of synergy strictly requires a statistically significant HA $\times$ SWE interaction term).

3.8 Leaf relative water content and its relationship to proline and chlorophyll responses

Biostimulant-induced changes in leaf relative water content (LRWC) revealed concentration-dependent modulation of plant water status, with implications for stress physiology. Humic acid applications progressively increased canopy leaf relative water content (LRWC) from 47.8% (control) to 50.0% (HA 3000 ppm) in pooled analysis (4.6% enhancement; CD 5% = 0.02), while seaweed extract produced smaller but significant improvements reaching 49.2% at maximum concentration (Tables 7 and 8). Notably, Year II exhibited consistently elevated LRWC values (51.8–52.4%) compared to Year I (43.8–47.6%) despite substantially reduced rainfall (718 vs. 1095 mm), suggesting that biostimulant-treated plants maintained proportionally greater transpirational capacity under severe drought stress. This apparent paradox—enhanced water vapor release under water-limited conditions—indicates fundamental shifts in hydraulic regulation

Table 7 Individual effect of foliar application of HA on RH (%) of custard apple cv. Balanagar

Treatments	I year (2022)	II year (2023)	Pooled (I and II year) (2022–2023)
T ₁ —Control	4.38	5.18	4.78
T ₂ —HA (1000 ppm)	4.39	5.20	4.80
T ₃ —HA (2000 ppm)	4.57	5.22	4.90
T ₄ —HA (3000 ppm)	4.76	5.24	5.00
SEm (±)	0.01	0.02	0.01
CD 5%	0.02	0.04	0.02

Table 8 Individual effect of foliar application of seaweed extract (SWE) on RH (%) of custard apple cv. Balanagar

Treatments	I year (2022)	II year (2023)	Pooled (I and II year) (2022–2023)
T ₁ —Control	4.46	5.13	4.79
T ₂ —SWE (1000 ppm)	4.54	5.22	4.88
T ₃ —SWE (2000 ppm)	4.54	5.21	4.88
T ₄ —SWE (3000 ppm)	4.55	5.27	4.92
SEm (±)	0.01	0.02	0.01
CD 5%	0.02	0.04	0.02

and stomatal function rather than simple stress-induced stomatal closure, consistent with improved water acquisition and allocation efficiency enabling sustained physiological activity.

Synergistic microclimate modifications in combined treatments demonstrated mechanistic integration across physiological scales. Treatment T15 (HA 3000 ppm + SWE 2000 ppm) achieved maximum LRWC (50.6%), representing 7.7% enhancement over control and significantly exceeding individual applications (Table 9; HA × SWE interaction CD 5% = 0.04). The correlation structure linking microclimate parameters to metabolic biomarkers revealed coordinated physiological regulation: relative humidity enhancement correlated inversely with proline accumulation ($r = -0.54$, $P < 0.01$) and positively with chlorophyll retention ($r = 0.61$, $P < 0.01$). This tripartite relationship suggests a mechanistic cascade wherein favourable microclimate conditions (reduced vapor pressure deficit) decrease transpirational demand, improving cellular water potential and reducing ABA-mediated stress signalling that would otherwise trigger proline biosynthesis and chlorophyll catabolism. The temporal dynamics support this interpretation: initial microclimate improvements (evident by August) preceded maximal metabolic responses (November–December), indicating that sustained favourable conditions enabled cumulative stress mitigation effects.

3.9 Principal component analysis of proline dynamics

Principal component analysis revealed distinct clustering patterns in proline accumulation across treatment groups and experimental periods (Fig. 1). PC1 and PC2 explained 58.3% and 24.7% of total variance (83.0%), respectively, demonstrating that the majority of variation in the measured proline and chlorophyll data could be captured by these two principal components. The biplot (Fig. 2) demonstrated clear temporal and treatment-dependent separation in stress metabolite dynamics.

Control treatment (T1) was positioned in the negative quadrant of PC1, indicating persistent stress conditions characterized by elevated proline concentrations throughout

Table 9 Interaction effect of foliar application of humic acid (HA) and seaweed extract (SWE) on RH (%) of custard apple cv. Balanagar

Treatments	I year (2022)	II year (2023)	Pooled (I and II year) (2022–23)
T ₁ – Control	4.32	5.09	4.70
T ₂ —HA (1000 ppm)	4.40	5.21	4.81
T ₃ —HA (2000 ppm)	4.43	5.16	4.80
T ₄ —HA (3000 ppm)	4.37	5.25	4.81
T ₅ —SWE (1000 ppm)	4.36	5.19	4.77
T ₆ —SWE (2000 ppm)	4.41	5.12	4.77
T ₇ —SWE (3000 ppm)	4.40	5.22	4.81
T ₈ —HA (1000 ppm) + SWE (1000 ppm)	4.39	5.28	4.84
T ₉ —HA (1000 ppm) + SWE (2000 ppm)	4.47	5.14	4.81
T ₁₀ —HA (1000 ppm) + SWE (3000 ppm)	4.57	5.29	4.94
T ₁₁ —HA (2000 ppm) + SWE (1000 ppm)	4.54	5.16	4.84
T ₁₂ —HA (2000 ppm) + SWE (2000 ppm)	4.70	5.29	4.99
T ₁₃ —HA (2000 ppm) + SWE (3000 ppm)	4.69	5.10	4.90
T ₁₄ —HA (3000 ppm) + SWE (1000 ppm)	4.79	5.24	5.01
T ₁₅ —HA (3000 ppm) + SWE (2000 ppm)	4.80	5.32	5.06
T ₁₆ —HA (3000 ppm) + SWE (3000 ppm)	4.76	5.28	5.02
SEm (±)	0.01	0.03	0.02
CD 5% (HA×SWE)	0.04	0.09	0.04

experimental duration. Treatment T13 (humic acid 2000 ppm plus seaweed extract 3000 ppm) exhibited the highest initial proline content, clustering in the positive region of PC1, suggesting an elevated stress response at the onset of the study. The combined treatment T15 (humic acid 3000 ppm plus seaweed extract 2000 ppm) effectively modulated proline concentrations, with vectors showing progression from initial elevated concentrations toward reduced final stress indicators along PC1.

Vector trajectories indicated that while individual humic acid and seaweed extract treatments (T4, T10, T7, T16) clustered near origin with moderate stress amelioration, the synergistic combination demonstrated superior stress mitigation efficacy. Separation of initial and final measurements along the PC1 axis reflects the temporal dynamics of biostimulant-mediated stress response modulation. Critical difference values of 0.39 for 2022 Final, 0.44 for 2023 Final, and 0.29 $\mu\text{mol g}^{-1}$ for Pooled Final measurements confirmed significant treatment effects on stress metabolite regulation in custard apple cv. Balanagar.

3.10 Metabolic relationships and stress response patterns

Correlation analysis revealed significant negative relationships between proline content and chlorophyll content across treatments and years (Pearson $r = -0.67$ to -0.73 , $P < 0.001$), indicating that conditions favoring lower stress-induced proline accumulation corresponded with enhanced chlorophyll retention (Fig. 3). This inverse relationship supports the interpretation that biostimulant-mediated improvements in plant water

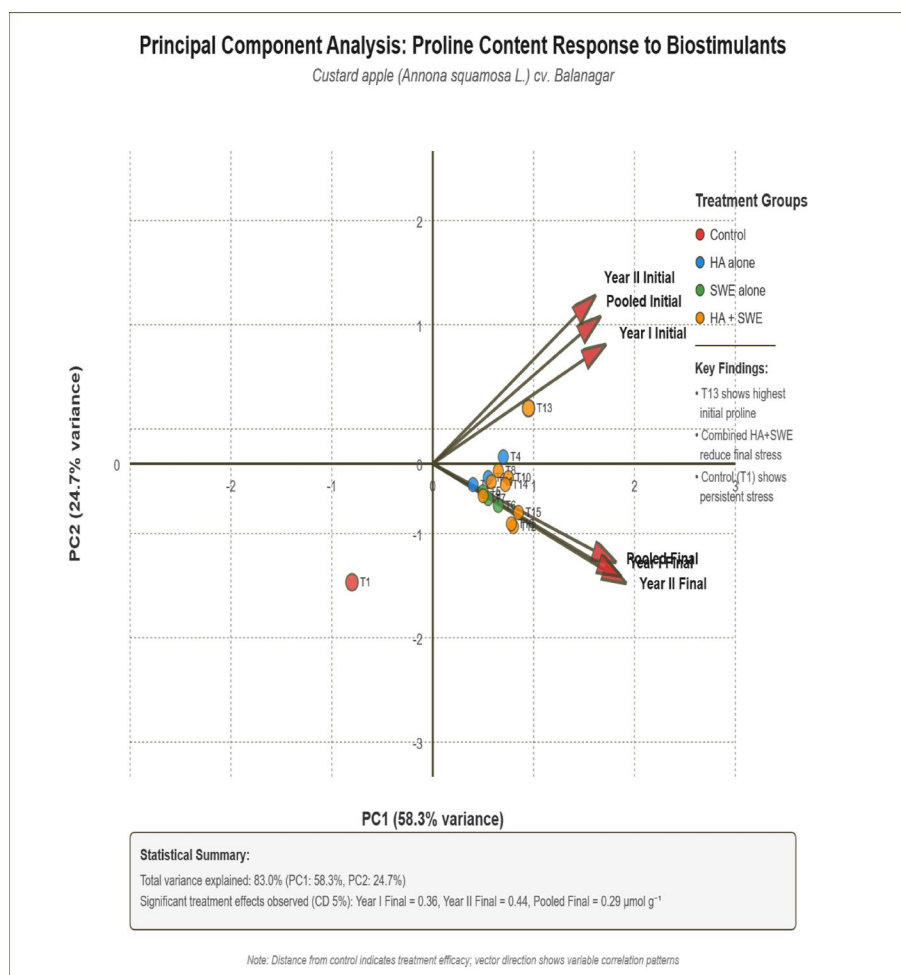


Fig. 1 Principal component analysis (PCA) biplot showing the clustering patterns of proline content across different treatments and experimental periods. PC1 and PC2 explain 58.3% and 24.7% of total variance (83.0%), respectively. Vectors represent proline measurements at initial and final sampling periods across Year I (2022), Year II (2023), and pooled data. Treatment codes: T1 (Control), T4 (HA 3000 ppm), T7 (SWE 3000 ppm), T10 (HA 1000 + SWE 3000 ppm), T13 (HA 2000 + SWE 3000 ppm), T15 (HA 3000 + SWE 2000 ppm), T16 (HA 3000 + SWE 3000 ppm). The separation of treatments along PC1 indicates differential stress mitigation efficacy, with T15 showing superior proline reduction

status simultaneously reduced osmotic stress signaling (lower proline) while maintaining photosynthetic capacity (higher chlorophyll). The magnitude of proline reduction (13.2% for the optimal treatment) exceeded chlorophyll enhancement (6.9%), suggesting that biostimulants may have primarily operated through stress mitigation rather than direct stimulation of photosynthetic machinery. While both values are statistically significant, it is important to acknowledge that these effect sizes are biologically modest. In the context of drought-stressed perennial fruit crops, reductions of 10–15% in osmotic stress markers have been associated with measurable improvements in physiological status in the literature [35]; however, whether these magnitudes translate to meaningful improvements in plant performance and yield in custard apple requires validation through productivity measurements in future studies. The modest chlorophyll increases likely reflect natural senescence-related degradation during reproductive development, which biostimulants partially attenuated but could not fully prevent. Treatment-specific patterns emerged in the relationship between initial and final measurements. High-efficacy

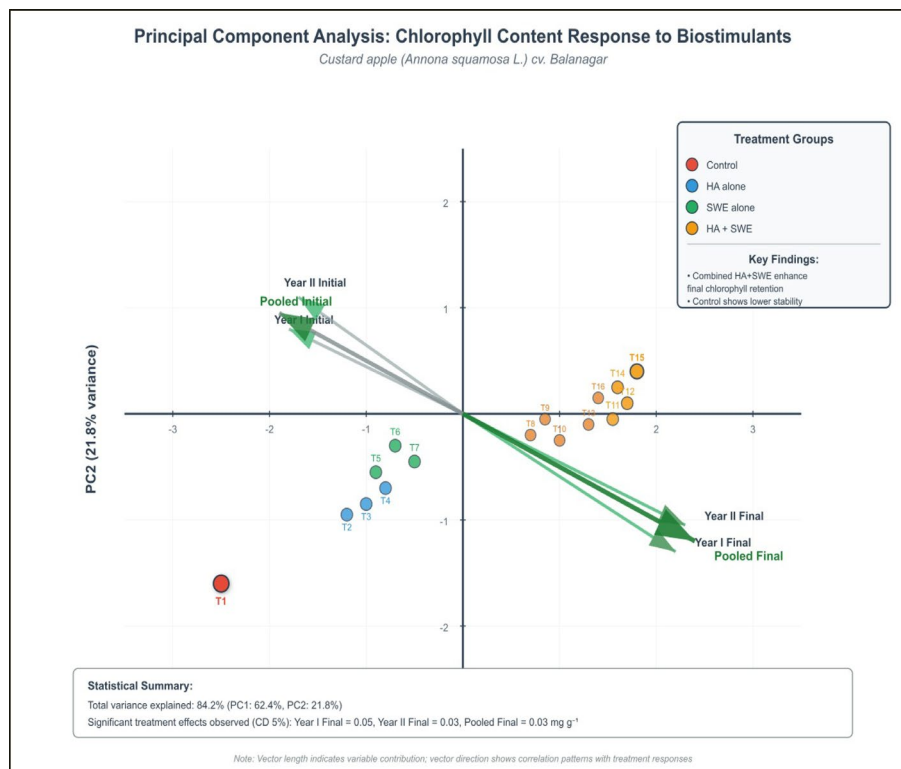


Fig. 2 Principal component analysis (PCA) biplot illustrating the relationships between chlorophyll content measurements across treatments and experimental years. The biplot demonstrates treatment clustering patterns and temporal dynamics of chlorophyll accumulation in response to biostimulant applications. Vectors indicate the direction and magnitude of variation for initial and final chlorophyll measurements in Year I, Year II, and pooled analysis

treatments (T15, T14, T12) showed the largest proline reductions ($5.5\text{--}6.2\ \mu\text{mol g}^{-1}$) combined with minimal chlorophyll loss ($0.01\text{--}0.04\ \text{mg g}^{-1}$), while control treatments exhibited moderate proline reduction ($4.6\ \mu\text{mol g}^{-1}$) but substantial chlorophyll degradation ($0.17\ \text{mg g}^{-1}$), indicating that effective stress management requires coordinated regulation of both osmotic adjustment and photosynthetic protection mechanisms.

4 Discussion

4.1 Biostimulant-mediated modulation of drought-responsive metabolism and chlorophyll stability in *Annona squamosa* under semi-arid field conditions

4.1.1 Drought-induced proline accumulation and stress signalling

Elevated proline concentrations observed during pre-treatment sampling in June ($13\text{--}16\ \mu\text{mol g}^{-1}$) reflect activation of stress-responsive metabolic pathways characteristic of water deficit conditions. Under drought stress, ABA-mediated signalling has been reported to upregulate P5CS gene expression and increase flux through the glutamate-P5C-proline biosynthetic pathway while inhibiting ProDH-mediated catabolism [41], these pathways were not directly measured in the present study [41]. The 15% higher baseline proline content in 2023 compared to 2022 quantitatively demonstrates this concentration-dependent stress response calibrated to drought intensity, corresponding with a 34% reduction in rainfall (718.30 mm vs. 1095 mm) and elevated pre-monsoon temperatures.

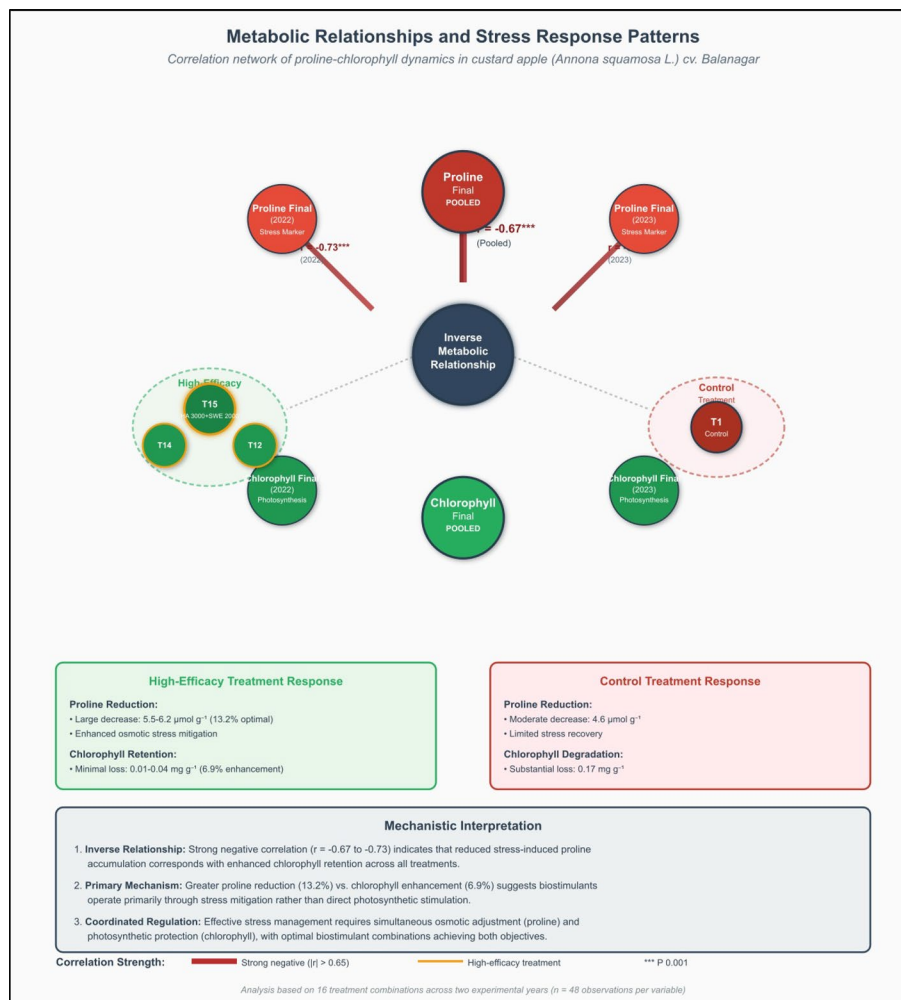


Fig. 3 Inverse proline-chlorophyll correlation reveals biostimulant-mediated stress mitigation in custard apple

Proline serves multiple protective functions under osmotic stress, acting as an osmo-protectant, a membrane stabilizer, and a free radical scavenger [21]. The substantially elevated initial values in both years confirm that custard apple experiences significant water deficit stress during pre-monsoon periods, establishing physiological conditions conducive to evaluating biostimulant-mediated stress mitigation.

4.1.2 Biostimulant-mediated modulation of proline metabolism

Foliar applications of humic acid and seaweed extract reduced final proline accumulation by 3.4–13.2% depending on treatment combination, indicating modulation of stress-response pathways rather than direct metabolic inhibition. The optimal combination of humic acid (3000 ppm) plus seaweed extract (2000 ppm) achieved 13.2% proline reduction, suggesting synergistic effects exceeding individual treatment efficacy. Three non-mutually exclusive mechanisms likely contribute to this response.

Enhanced water relations, reducing stress signalling intensity, represent the primary mechanism. Humic acid-mediated enhancement of root hydraulic conductivity and soil water availability occurs through improved soil structure, enhanced root system architecture via auxin-like activity, and increased plasma membrane H^+ -ATPase activity that

energizes nutrient and water uptake [10]. Seaweed extract-derived osmoprotectant-improves cellular water retention, collectively reducing ABA accumulation and downstream stress-responsive gene expression. Studies in tomato demonstrate that seaweed extract application reduces ABA content by 15–25% under drought stress while maintaining turgor pressure [49], consistent with observed proline reduction patterns in our study.

Enhanced antioxidant capacity provides a secondary mechanism. Both humic acid and seaweed extract increase activities of ROS-scavenging enzymes, including SOD, CAT, and APX, reducing oxidative stress that independently triggers proline biosynthesis [17, 32]. The P5CS promoter contains stress-responsive elements activated by H_2O_2 signalling, enhanced antioxidant capacity dampens this oxidative trigger [29]. The reduction in proline accumulation in treated plants suggests decreased oxidative stress burden and improved cellular homeostasis.

Nitrogen partitioning represents a third contributing mechanism. Proline biosynthesis competes with chlorophyll synthesis for glutamate precursors. By improving overall nitrogen status through enhanced uptake and assimilation, biostimulants may reduce the proportion of nitrogen diverted to stress metabolism, explaining coordinated proline reduction with enhanced chlorophyll retention. This interpretation is supported by the strong inverse correlation between proline content and chlorophyll content ($r = -0.67$ to -0.73 , $P < 0.001$).

4.1.3 Concentration-dependent efficacy and synergistic interactions

Individual humic acid application reduced proline accumulation in a concentration-dependent manner, with the magnitude increasing progressively from 4.4% (1000 ppm) to 5.0% (3000 ppm) in pooled analysis. Similarly, seaweed extract showed dose–response efficacy, though optimal chlorophyll enhancement occurred at 2000 ppm with a slight decline at 3000 ppm, indicating potential saturation of receptor sites or feedback inhibition of signalling pathways previously reported in hormone-like biostimulant applications [35].

Combined treatments demonstrated superior efficacy compared to individual applications, with treatment T15 (humic acid 3000 ppm plus seaweed extract 2000 ppm) achieving optimal physiological performance. This synergistic interaction likely reflects complementary mechanisms: humic acid primarily enhances root development and nutrient uptake capacity while seaweed extract provides phytohormone regulation and antioxidant activation. The consistent treatment effects despite inter-annual rainfall variation (34% reduction in 2023) demonstrate robust stress mitigation across varying drought intensities.

4.1.4 Chlorophyll retention and photosynthetic protection

Biostimulant applications enhanced final chlorophyll content by up to 6.9% compared to control, with a smaller magnitude of improvement relative to proline reduction (13.2%). This differential response is consistent with the interpretation that biostimulants may primarily operate through stress mitigation rather than direct stimulation of photosynthetic machinery, though this interpretation remains tentative without direct mechanistic measurements. Chlorophyll degradation during reproductive development reflects

natural senescence processes that biostimulants partially delayed but could not fully prevent.

The cytokinin content in seaweed extracts delays leaf senescence and maintains photosynthetic capacity under stress conditions [12]. Humic acids similarly promote photosynthetic efficiency through improved nutrient availability and stomatal regulation [10]. The coordinated enhancement of chlorophyll retention with proline reduction indicates improved overall plant water status rather than independent metabolic effects. High-efficacy treatments (T15, T14, T12) showed largest proline reductions ($5.5\text{--}6.2\ \mu\text{mol g}^{-1}$) combined with minimal chlorophyll loss ($0.01\text{--}0.04\ \text{mg g}^{-1}$), while control exhibited moderate proline reduction ($4.6\ \mu\text{mol g}^{-1}$) but substantial chlorophyll degradation ($0.17\ \text{mg g}^{-1}$).

4.1.5 Metabolic coordination and stress response patterns

Principal component analysis revealed distinct treatment clustering patterns, with combined treatments separating from individual applications and control along the PC1 axis. The separation of initial and final measurements along PC1 reflects temporal dynamics of biostimulant-mediated stress response modulation. High-efficacy treatments clustered in the positive PC1 region for chlorophyll parameters and the negative PC1 region for proline parameters, demonstrating coordinated metabolic regulation.

The inverse relationship between proline and chlorophyll content indicates that conditions favouring lower stress-induced proline accumulation correspond with enhanced chlorophyll retention. This coordination suggests that effective stress management requires simultaneous regulation of osmotic adjustment and photosynthetic protection mechanisms. The reduced proline accumulation in biostimulant-treated plants is interpreted as reflecting improved plant water status that decreased the metabolic requirement for osmolyte accumulation; however, the dual role of proline as both a stress indicator and a protective metabolite means that alternative interpretations cannot be excluded without direct stress marker measurements.

4.1.6 Agronomic implications and future perspectives

These findings support a physiological basis for biostimulant application in custard apple production under semi-arid conditions. The optimal treatment combination (humic acid 3000 ppm plus seaweed extract 2000 ppm) provides an actionable recommendation for orchard management, though economic analysis and yield validation remain necessary. The progressive dose–response relationship suggests that humic acid concentrations approaching 3000 ppm optimize foliar coverage and cellular uptake capacity, though the absence of plateau effects indicates potential for further optimization within economically viable ranges.

4.1.7 Mechanisms of chlorophyll retention under stress

Biostimulant-mediated enhancement of chlorophyll content reflects multiple coordinated mechanisms. Enhanced nitrogen availability stimulates chlorophyll synthesis by upregulating genes encoding magnesium chelatase subunits (CHLH, CHLI, CHLD), which catalyse the rate-limiting step of inserting Mg^{2+} into protoporphyrin IX during chlorophyll biosynthesis [22, 26]. Humic acids increase nitrogen availability through improved mineralization and enhanced nitrate uptake via increased plasma membrane

H⁺-ATPase activity [10]. The 2.09% chlorophyll increase with humic acid at 3000 ppm reflects this enhanced nitrogen nutrition.

Protection against chlorophyll degradation provides an additional mechanism. In the literature, biostimulants have been associated with downregulation of chlorophyll catabolic genes (NYC1, SGR, PAO), which would be consistent with the chlorophyll retention observed here, though gene expression was not assessed in the present study [50]. Cytokinin content in seaweed extracts suppresses senescence-associated gene expression and maintains chloroplast structural integrity during reproductive growth [9]. The optimal chlorophyll enhancement with seaweed extract at 2000 ppm (2.45 mg g⁻¹) compared to 3000 ppm (2.42 mg g⁻¹) suggests an optimal cytokinin concentration threshold, beyond which receptor desensitization may occur.

Enhanced antioxidant capacity protects the photosynthetic apparatus from oxidative damage. ROS generated during photosynthetic electron transport under stress directly oxidize chlorophyll molecules and degrade photosystem proteins. Biostimulant-enhanced antioxidant enzyme activities (SOD, CAT, APX) mitigate this damage, maintaining chlorophyll stability [17]. Despite these protective mechanisms, slight chlorophyll declines from initial to final measurements occurred across all treatments, reflecting natural senescence associated with reproductive development and nutrient remobilization during fruit maturation. However, biostimulant-treated plants maintained significantly higher chlorophyll concentrations throughout this critical period, indicating delayed senescence and greater potential photosynthetic capacity (noting that actual photosynthetic performance would require gas exchange or fluorescence measurements to confirm).

4.2 Synergistic interactions and complementary mechanisms

Superior performance of treatment T15 (humic acid 3000 ppm plus seaweed extract 2000 ppm) in reducing proline accumulation (13.2% reduction) while enhancing chlorophyll content (6.9% increase) demonstrates significant synergistic interaction. This synergy operates through complementary mechanisms: humic acids primarily enhance nutrient availability and root system development through improved soil–plant interactions, while seaweed extracts provide direct hormonal signals and bioactive compounds modulating plant metabolism [28]. The combination provides both improved resource acquisition capacity (humic acid-mediated) and enhanced resource utilization efficiency (seaweed extract-mediated).

Temporal dynamics contribute to synergistic efficacy. Humic acids exhibit persistent effects on soil properties and root architecture, providing sustained benefits throughout the growing season. Seaweed extracts, containing readily available hormones and signalling molecules, produce more immediate metabolic responses. The combination of sustained root-level improvements with periodic foliar metabolic stimulation creates optimal conditions for stress tolerance.

Non-additive patterns emerged in certain combinations. Treatment T13 (humic acid 2000 ppm plus seaweed extract 3000 ppm) showed higher proline content (9.09 μmol g⁻¹) than some individual treatments, suggesting complex biochemical interactions and potential antagonistic effects at certain concentration combinations, possibly due to excessive hormonal signalling causing transient stress responses. The optimal synergistic effect achieved with humic acid 3000 ppm plus seaweed extract 2000 ppm,

rather than the highest concentration combination (3000 ppm plus 3000 ppm) supports optimal concentration thresholds rather than simple dose–response linearity.

4.3 Concentration–response relationships and biological thresholds

Concentration-dependent responses reveal important considerations for practical application. Humic acid showed progressive improvement with increasing concentration from 1000 to 3000 ppm without a plateau effect at the highest concentration tested, suggesting potential optimization at concentrations equal to or slightly higher than 3000 ppm, though economic considerations require evaluation of cost–benefit ratios.

Seaweed extract exhibited peak efficacy at 2000 ppm for chlorophyll content with slightly diminished response at 3000 ppm. This non-linear pattern indicates an optimal biological activity window, likely related to hormone receptor saturation or activation of negative feedback mechanisms at excessive concentrations. Such biphasic dose–response curves are characteristic of hormone-like substances and have been extensively documented in plant growth regulator research [35]. From a practical perspective, treatment T15 represents an optimal balance between physiological efficacy and input costs, achieving superior results without requiring the highest concentration of both biostimulants.

4.4 Proposed mechanistic framework for biostimulant-mediated stress mitigation

The physiological responses observed in this study are summarized in a hypothetical conceptual framework (Fig. 4—Proposed Hypothetical Model; Not Experimentally Validated in This Study) that draws on the literature to suggest possible multi-level regulatory mechanisms. This framework is intended as a hypothesis-generating tool for future mechanistic research and should not be interpreted as experimentally demonstrated in the present study. Biostimulant applications interrupt stress signalling cascades at multiple nodes: humic acid-enhanced root hydraulic conductivity and nutrient acquisition improve plant water status (evidenced by 7.7% leaf relative water content (LRWC) increase), reducing ABA-mediated stress signalling intensity, while seaweed extract-derived cytokinins and antioxidant compounds directly suppress senescence-associated gene expression and mitigate oxidative damage. These complementary mechanisms converge to downregulate P5CS-mediated proline biosynthesis (13.2% reduction) while simultaneously enhancing chlorophyll retention (6.9% increase) through reduced nitrogen competition for glutamate precursors and delayed chlorophyll catabolic enzyme activation. The strong inverse correlation between proline content and chlorophyll concentration ($r = -0.73$, $P < 0.001$) supports coordinated metabolic regulation rather than independent pathway effects. While direct measurements of gene expression (P5CS, ProDH, CHLH, NYC1), antioxidant enzyme activities (SOD, CAT, APX), and phytohormone concentrations (ABA, cytokinins) were not performed in the current study—representing important targets for future molecular investigations—the observed phenotypic responses align with established stress physiology paradigms and provide a testable framework for mechanistic dissection of biostimulant efficacy in perennial fruit crops under water-limited conditions.

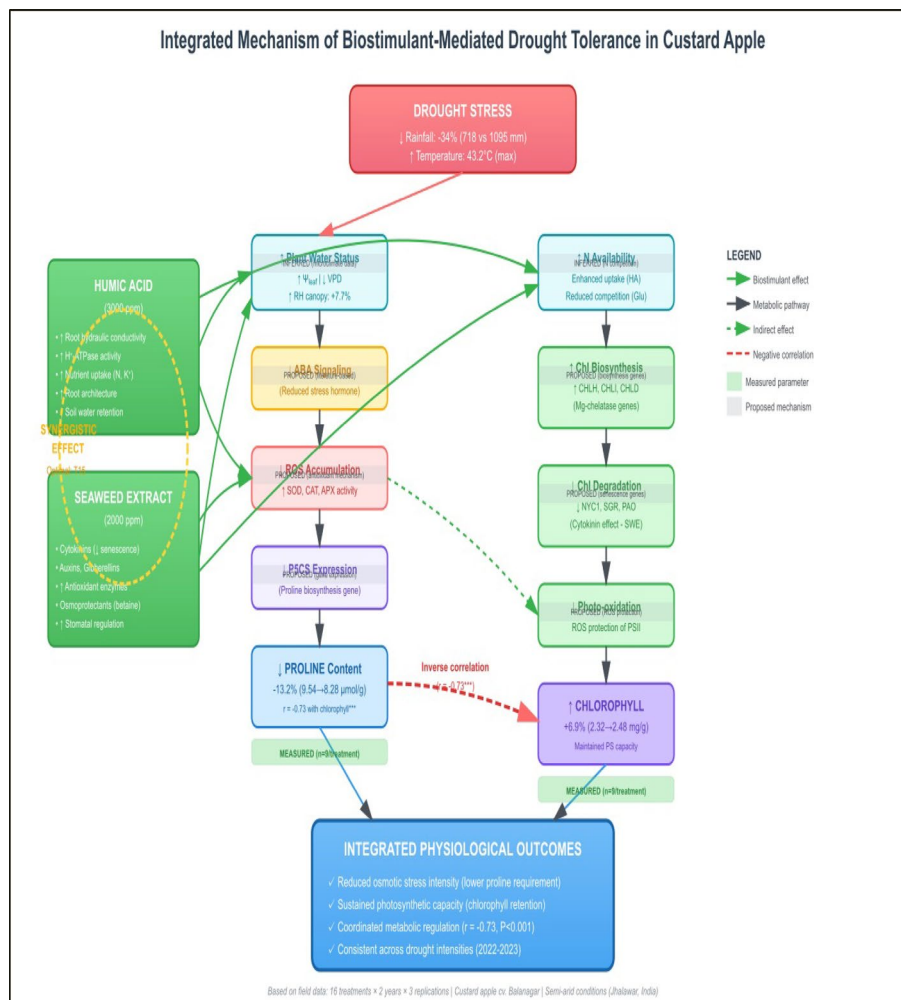


Fig. 4 Conceptual model summarizing proposed mechanisms (ABA–ROS–P5CS–Proline–Chlorophyll linkage)

4.5 Research limitations and mechanistic knowledge gaps

While this study demonstrates clear biostimulant effects on stress-responsive metabolic markers, several mechanistic questions remain unresolved. Proline concentrations were measured at two time points, but metabolic flux rates and enzyme activities (P5CS, ProDH) were not quantified, limiting insights into whether biostimulants affect biosynthesis, catabolism, or both processes. Future studies employing ^{13}C or ^{15}N isotope labeling could trace proline turnover dynamics and identify specific regulatory nodes.

Chlorophyll measurements assessed total content but not functional photosynthetic capacity. Complementary measurements of chlorophyll fluorescence parameters (F_v/F_m , ΦPSII , NPQ) and gas exchange rates would distinguish between maintained chlorophyll content with impaired function versus preserved photosynthetic capacity. The 6.9% chlorophyll enhancement may translate to larger or smaller photosynthetic improvements depending on photosystem functionality.

Molecular mechanisms linking biostimulant application to observed metabolic changes require elucidation. Gene expression profiling of stress-responsive transcription factors (DREB, NAC, MYB families), biosynthetic enzymes (P5CS, CHLH, GS/GOGAT), and hormone metabolism genes would identify regulatory pathways activated

by biostimulant treatments. Proteomic and metabolomic approaches could reveal post-transcriptional regulation and metabolite network responses. The lack of genomic resources for *Annona* species limits molecular investigation, highlighting the need for transcriptome sequencing and functional gene annotation in this underutilized crop.

Several important aspects were beyond the scope of the present study and represent priorities for future research. Root system architecture and rhizosphere processes—including root growth, mycorrhizal colonization, and rhizosphere microbial community structure—were not assessed; these below-ground processes are particularly relevant given that humic acid is known to act primarily through root-zone effects, and their quantification would provide valuable mechanistic insights. Molecular and enzymatic parameters (gene expression, antioxidant enzyme activities, ABA and cytokinin concentrations) were not measured and should be incorporated in future studies. Long-term investigations evaluating cumulative effects of repeated annual applications on soil health and tree productive lifespan, as well as extension of the approach to other commercially cultivated *Annona* species (*A. atemoya*, *A. cherimola*, *A. muricata*), would broaden the applicability of these findings.

4.6 Implications for custard apple production systems

These findings establish a physiological basis for biostimulant integration in custard apple orchards under water-limited conditions. The optimal treatment combination (humic acid 3000 ppm plus seaweed extract 2000 ppm) applied during flowering and fruit set stages provides an actionable recommendation for orchard management. The consistent efficacy across contrasting rainfall years (1095 mm vs. 718 mm) demonstrates treatment robustness under variable environmental conditions characteristic of semi-arid production regions.

Integration with yield parameters, fruit quality attributes (sugar content, firmness, shelf life), and economic analyses is essential for comprehensive validation of commercial viability. The present study did not include growth, yield, or fruit quality measurements, which represents a significant limitation in assessing the practical agronomic relevance of the observed physiological responses. Future investigations should incorporate these productivity metrics alongside physiological markers to provide a complete evaluation of biostimulant efficacy. Cost–benefit analysis should consider biostimulant input costs, application labour, and potential productivity gains to determine economic feasibility for small-holder and commercial orchards. Future investigations should examine application timing optimization, frequency effects, and integration with conventional nutrient management programs to develop holistic orchard management protocols enhancing both environmental sustainability and economic productivity.

5 Conclusion

This 2-year field investigation demonstrates that foliar-applied biostimulants significantly modulate stress-responsive metabolism and photosynthetic capacity in custard apple cv. Balanagar under semi-arid conditions. Combined application of humic acid (3000 ppm) and seaweed extract (2000 ppm) achieved optimal physiological performance, reducing proline accumulation by 13.2% ($8.28 \mu\text{mol g}^{-1}$) while enhancing chlorophyll retention by 6.9% (2.48 mg g^{-1}) relative to untreated controls. Individual applications showed beneficial effects, though quantitatively inferior to synergistic combinations.

The inverse correlation between proline content and chlorophyll retention ($r = -0.67$ to -0.73 , $P < 0.001$) indicates that biostimulants primarily operate through stress mitigation rather than direct photosynthetic stimulation. The greater magnitude of proline reduction compared to chlorophyll enhancement supports this mechanistic interpretation. Principal component analysis confirmed distinct treatment-dependent metabolic clustering, with PC1 and PC2 explaining 83% of variance in proline dynamics and 84.2% in chlorophyll parameters, demonstrating coordinated regulation of osmotic adjustment and photosynthetic protection.

The superior performance of combined treatments is consistent with complementary mechanisms reported in the literature, involving HA-mediated improvements in water and nutrient relations and SWE-associated hormonal and antioxidant effects; however, these mechanisms were not directly measured. Treatment consistency across contrasting rainfall years (1095 mm vs. 718 mm) suggests robustness under variable environmental stress. These findings provide a physiological basis for further investigation of biostimulant applications in drought-prone custard apple production systems, with the important caveat that integration with yield, fruit quality, and economic analyses is necessary for comprehensive agronomic validation.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44372-026-00681-2>.

Supplementary Material 1

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Author contributions

Anju Yadav: Conceptualization, methodology, investigation, formal analysis, writing—original draft, visualization. Prerak Bhatnagar: Investigation, data curation, formal analysis, writing—review and editing. Jitendra Singh: Supervision, resources, project administration. C.K. Arya: Methodology, validation, writing—review and editing. Kavita Aravindakshan: Resources, writing—review and editing. Anil Kumar Gupta: Conceptualization, methodology, writing—review and editing, Supervision. All authors approved the final manuscript.

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Data availability

All data generated and analyzed during this study are included in this published article and its supplementary information files. Raw data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Plant material collection and experimental procedures complied with institutional guidelines of the College of Horticulture and Forestry, Jhalawar, Rajasthan, India, and national guidelines under the Biological Diversity Act, 2002 of India. The custard apple (*Annona squamosa* L.) cv. Balanagar trees used in this study were cultivated specimens maintained under commercial orchard management at the research farm of the College of Horticulture and Forestry, Jhalawar (24.5939° N, 76.1643° E). No specific permissions or licenses were required for the use of these cultivated plant materials as they were grown within institutional research facilities for agricultural research purposes. No endangered or protected plant species were involved in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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