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METABOLIC RESPONSES OF TOBACCO (*NICOTIANA* SPP.) UNDER WATERLOGGING STRESS: A SYSTEMATIC REVIEW

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SUMMARY

Waterlogging represents one of the most destructive abiotic stresses, preventing global crop production, including tobacco (*Nicotiana* spp.), which experiences yield reductions reaching 83% under extended flooding conditions. This systematic review aimed to determine the tobacco-specific synthesis that combines metabolomic data from different genotypes with an assessment of proposed metabolic markers and identification of important research areas that require addressing in future investigations. This systematic review synthesizes findings from 18 peer-reviewed articles, which researchers selected from 571 records that the Scopus and Web of Science databases identified during the period from 2018 to 2024. The compiled evidence shows how waterlogging triggers tobacco plants to make controlled metabolic changes, affecting their antioxidant defense systems, anaerobic energy production, nitrogen-containing compound synthesis, and cellular communication pathways. Tolerant genotypes consistently exhibited enhanced antioxidant and fermentation enzyme activities and greater energy reserve retention than susceptible genotypes. Identifying enzymatic activities, energy-related metabolites, and Ca^{2+} ions was recurrent as candidate metabolic indicators.

Keywords: Tobacco (*Nicotiana* spp.), waterlogging, inundation stress, plant defense systems, metabolite profile, antioxidant enzymes, metabolic indicators

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Key findings: According to the literature review, tolerant tobacco (*Nicotiana* spp.) genotypes maintained elevated levels of antioxidant and fermentation enzymes, higher energy reserves and ATP levels, and increased Ca^{2+} ion concentrations. These collectively serve as promising metabolic indicators for developing waterlogging-tolerant cultivars through breeding programs.

INTRODUCTION

Tobacco (*Nicotiana* spp.) is a high-value global commercial crop that supports the livelihood of farming communities, especially in Asia and Africa, and considerably contributes to national incomes through export revenues (Vellios *et al*., 2018). However, the tobacco crop has recently faced harsh climatic conditions, such as waterlogging, indicating an association with reduced plant growth, development, and productivity (Nurjani *et al*., 2020). Waterlogging can reduce the tobacco yield by 83% if leaving the plants in water for a longer period (Habib *et al*., 2022).

Under waterlogging, oxygen deprivation in the root zone triggers metabolic perturbations affecting both primary and secondary metabolite profiles. Studies have proposed antioxidant metabolites, stress-responsive compounds, and phytohormones as candidate indicators for flooding tolerance (Yoo *et al*., 2024). Documentation of considerable variation in waterlogging survival among tobacco cultivars has succeeded (Habib *et al*., 2022; 2024), with tolerant genotypes characterized by enhanced root formation, greater antioxidant capacity, and activation of stress-responsive gene networks (Chen *et al*., 2019; Hu *et al*., 2023). Metabolomics has emerged as a powerful tool for elucidating the biochemical basis of flooding tolerance (Carrera *et al*., 2021; Tyagi *et al*., 2023).

The impact of waterlogging stress results in both primary and secondary metabolite profiles, as revealed through analyses of protein and phenolic compounds actively participating in plant defense systems (Jaiswal and Srivastava, 2018; Pan *et al*., 2021). Despite these advances, significant knowledge gaps remain regarding the specific metabolic signatures distinguishing waterlogging-tolerant from susceptible tobacco genotypes. The research before this study has scrutinized single biological measurements, while no current research has examined all metabolic measurements across different genetic backgrounds. The industry lacks dependable metabolic markers that can be applicable to conduct fast testing of tobacco genetic resources. Multiple studies have probed how plants survive waterlogged conditions through their various mechanisms of waterlogging resistance. The research conducted by Pan *et al*. (2021) presented a complete assessment of development changes in plants through their physical appearance and internal body functions and genetic changes. However, they did not study how plants metabolize their substances or how they react to tobacco-specific conditions. Tyagi *et al*. (2023) reviewed plant responses to abiotic stress through their metabolomic studies, but they did not present results for specific plant genetic variations. The two reviews conducted research on two different plant types, but these did not develop complete metabolic assessment methods, which could be beneficial to create new breeding techniques. The following review fills these gaps by critically synthesizing tobacco-specific metabolomic evidence, evaluating indicator reliability, and advancing a multi-indicator approach for breeding applications.

Tobacco (*Nicotiana* spp.) serves as an effective model for waterlogging metabolomic research because different tobacco plants demonstrate various levels of flood protection, which extends from Bojonegoro-1 high resistance to Kemloko-1 and Prancak-95 low resistance (Nurhidayati *et al*., 2017; Habib *et al*., 2024). The organism establishes its role as a transgenic host to create cross-species validation tests because its nicotine biosynthetic pathway combines with GABA (gamma-aminobutyric acid)-mediated hypoxic signaling (Zhang *et al*., 2016), and its established function as a transgenic host for cross-species validation (Pan *et al*., 2019; Xiong *et al*., 2024) provides extensive

Table 1. PICO components of this research.

P (Population)	I (Intervention)	C (Comparison)	O (Outcome)	C (Context)
Tobacco	Waterlogging, Profile metabolite	Normal versus waterlogged conditions, tolerant versus sensitive genotypes.	Changes in secondary metabolite profiles, Differences in primary and secondary metabolite profiles, Biomarker identification	

applications. The presence of secondary plant metabolite profile changes has proved effective for the identification of breeding programs' biochemical indicators (Herlina *et al*., 2025).

This review aimed to a) characterize tobacco metabolic profiles under waterlogging based on published evidence, b) compare metabolic responses of tolerant and susceptible genotypes, and c) evaluate candidate metabolic indicators for breeding programs. The synthesis will use metabolomic and genotypic data from reviewed literature to demonstrate biochemical pathways, leading to waterlogging tolerance while creating a scientific basis to develop flood-tolerant cultivars.

MATERIALS AND METHODS

The development of a systematic literature search strategy succeeded using the PICO framework (population, intervention, comparison, and outcome), with a single keyword string constructed to restrict the database search (Table 1). The systematic literature search commenced on January 10, 2025, using the following Boolean search string in both Scopus and Web of Science: ("tobacco" OR "*Nicotiana*") AND ("waterlogging" OR "flooding stress" OR "submergence" OR "drained stress") AND ("metabolite profiling" OR "metabolic fingerprinting" OR "metabolic profiling" OR "metabolite analysis" OR "metabolomics" OR "biomarker").

The upload of search metadata proceeded to the screening stage at https://parsif.al/ for duplicate removal and filtration based on predefined inclusion and exclusion criteria. The inclusion criteria comprised English-language, original research articles published from 2018 to 2024 with full-text accessibility, while excluding non-English articles, inaccessible full texts, book chapters, review articles, and pre-2018 publications. Applying the restriction to 2018–2024 sought to focus on recent methodological advances in metabolomics platforms (e.g., GC-MS, LC-MS, and NMR) and the most current understanding of tobacco waterlogging responses, as earlier studies relied predominantly on single-parameter physiological assays with limited metabolomic resolution. The selected articles include information from earlier fundamental research studies through their direct citations.

Ensuring critical depth entailed evaluation of each selected article for its findings and its methodological rigors, including experimental design, the number of genotypes tested, duration and severity of waterlogging treatments, analytical platforms used (e.g., GCMS, LCMS, and HPLC), and statistical approaches. Inconsistencies among studies regarding metabolite quantification methods and stress application protocols gained systematic notations and discussion in the results section. This approach distinguishes the present review from descriptive compilations by providing a critical assessment of the evidence base (Carrera *et al*., 2021).

RESULTS AND DISCUSSION

The PRISMA flow diagram shows how selections progressed. From 571 articles, the removal of 76 duplicates ensued. The screening process resulted in the elimination of 433 records. Of 62 retrieved, 27 were inaccessible. The assessment process evaluated 35 items and excluded 17 of them. The research team selected 18 studies for their

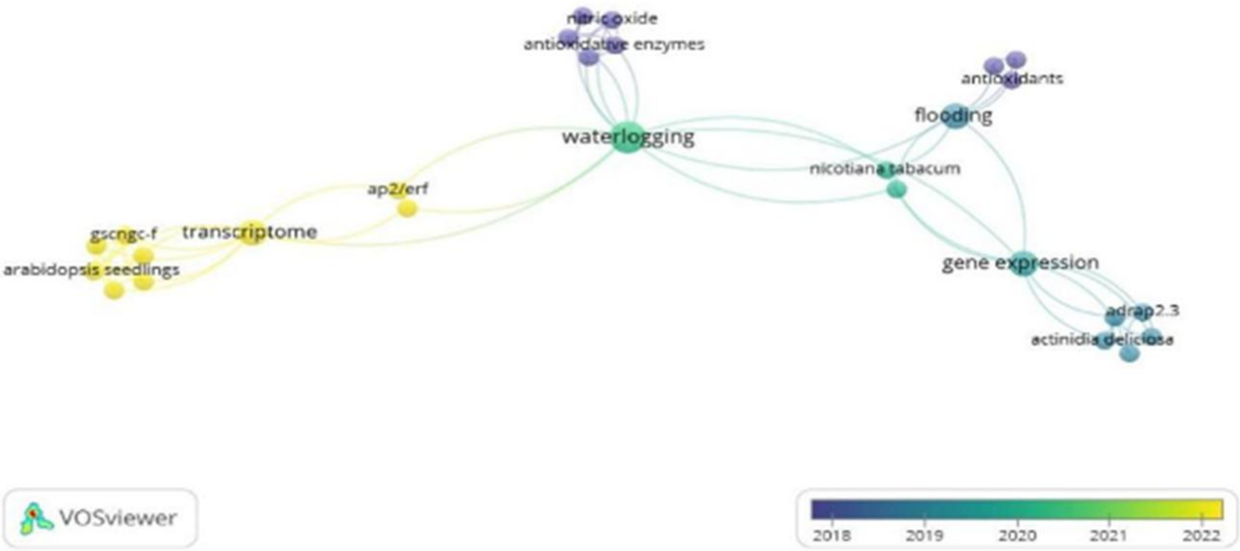

Figure 1. Map of research development on tobacco metabolites tolerant to waterlogging.

study. The VOS viewer bibliometric map (Figure 1) showed topic emergence from 2018 to 2022, with waterlogging as the central node linking flooding, gene expression, and antioxidant research.

Metabolite profile of tobacco under waterlogging stress

This reviewed literature offers extensive information on the variations of the metabolite profiles in the tobacco plants by exposing them to waterlogging stress conditions. It also explored their coordinated adaptive strategies to counter the oxidative stress, oxygen deprivation, and metabolic disturbances that mostly occur in the prolonged flooded conditions (Table 2). The antioxidant defense systems, energy metabolism, nitrogen compounds, and cellular signaling were the metabolic systems that revealed major variations (Jaiswal and Srivastava, 2018; Pan *et al*., 2021; Gao *et al*., 2023), with their integration associated with waterlogging tolerance development.

However, antioxidant enzymes' reliability as tolerance indicators requires careful evaluation. Jaiswal and Srivastava (2018) reported remarkable increases in antioxidant enzyme activities, including catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX), and polyphenol oxidase (PPO), with the largest increment in tolerant genotypes. Though antioxidant enzymes' activities remained high in tolerant genotypes, research discovered substantial differences because of different stress lengths and developmental stages and methodology (Hasanuzzaman *et al*., 2020; Gao *et al*., 2023). Non-enzymatic antioxidants, such as ascorbic acid and phenolic compounds, declined, though less in tolerant genotypes. Low molecular weight (LMW) antioxidants, including ascorbic acid, glutathione, and tocopherol, function as effective ROS scavengers (Yemelyanov *et al*., 2020). The AsA-GSH cycle shows decreased dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities under prolonged waterlogging, indicating potential loss of sustained antioxidant capacity (Hasanuzzaman *et al*., 2020).

Waterlogging leads to hypoxia, which causes cells to begin using different energy pathways instead of their normal energy pathways. The plant's oxidative phosphorylation process stops working when there is insufficient oxygen, and the plant starts using its glycolytic and fermentative pathways. The process of fermentation relies

Table 2. Metabolite profile variations in antioxidant, energy, and signaling pathways under hypoxic conditions.

Metabolite group	Metabolite type	Profile variations	Citations
1. Antioxidant System			
Antioxidant Enzymes	CAT, POX, SOD, APX	Significant increase, particularly in tolerant genotypes	Jaiswal and Srivastava (2018)
Non-enzymatic Antioxidants	Ascorbic acid, phenol	Decreased levels, less pronounced decline in tolerant genotypes	Jaiswal and Srivastava (2018)
Low Molecular Weight Antioxidants	Ascorbate, glutathione, tocopherol	Active participation in ROS degradation	Yemelyanov *et al*. (2020)
2. Energy Metabolism			
Fermentation Enzymes	PDC, ADH	Enhanced activity in tolerant transgenic plants	Pan *et al*. (2019)
Energy Compound	ATP	Reduced levels, higher retention in tolerant plants	Phukan *et al*. (2018)
Carbohydrates	Non-structural carbohydrates, total soluble carbohydrates	Progressive decline, higher levels maintained in tolerant plants	Li *et al*. (2022)
3. Organic Acids and Primary Metabolites			
Organic Acids	Pyruvate, succinic acid	Significant accumulation in waterlogging-tolerant cells	Lee *et al*. (2014)
Amino Acids	Alanine	Predominant (60% of total amino acids) under hypoxic conditions	Lee *et al*. (2014)
4. Proteins and Nitrogen Compounds			
Specific Proteins	20 kDa protein, 43 kDa protein	Expressed in waterlogging-tolerant genotypes	Jaiswal and Srivastava (2018)
Osmolytes	Proline	Increased accumulation in waterlogging-tolerant crops	Xiong *et al*. (2024)
5. Signaling Compounds and Stress Markers			
Signaling Ion	Ca^{2+}	Elevated levels, higher in tolerant plants	Li *et al*. (2022)
Oxidative Stress Markers	ROS, MDA	Reduced accumulation in tolerant transgenic plants	Bai *et al*. (2023)

mainly on two enzymes—pyruvate decarboxylase and alcohol dehydrogenase (ADH)—to make this transformation happen. The PDC (pyruvate dehydrogenase complex) enzyme changes pyruvate into acetaldehyde, which the ADH enzyme converts into ethanol, while producing NAD^+, allowing glycolysis to proceed in the absence of oxygen (Pan *et al*., 2019; Pan *et al*., 2021). PDC and ADH enzymes show increased activity in plants, leading to better protection against waterlogging (Gao *et al*., 2023). The research carried out by Phukan *et al*. in 2018 demonstrated that tolerant genotypes maintain higher ATP levels because they effectively use starch reserves. The research conducted by Li *et al*. in 2022 showed that tolerant cultivars maintained higher carbohydrate reserves while their carbohydrate stores decreased. The anaerobic environment created the conditions for alanine to reach its highest level of accumulation (Lee *et al*., 2014; Rocha *et al*., 2010).

Jaiswal and Srivastava (2018) identified specific proteins (20 kDa and 43 kDa) in rice-tolerant cultivars activating molecular defense systems. Tolerant genotypes maintained higher Ca^{2+} ion concentrations (Li *et al*., 2022), while tolerant plants exhibited lower ROS and malondialdehyde (MDA) levels (Bai *et al*., 2023). The waterlogging-tolerant genotypes show increased proline levels, which provide osmotic protection; however, their

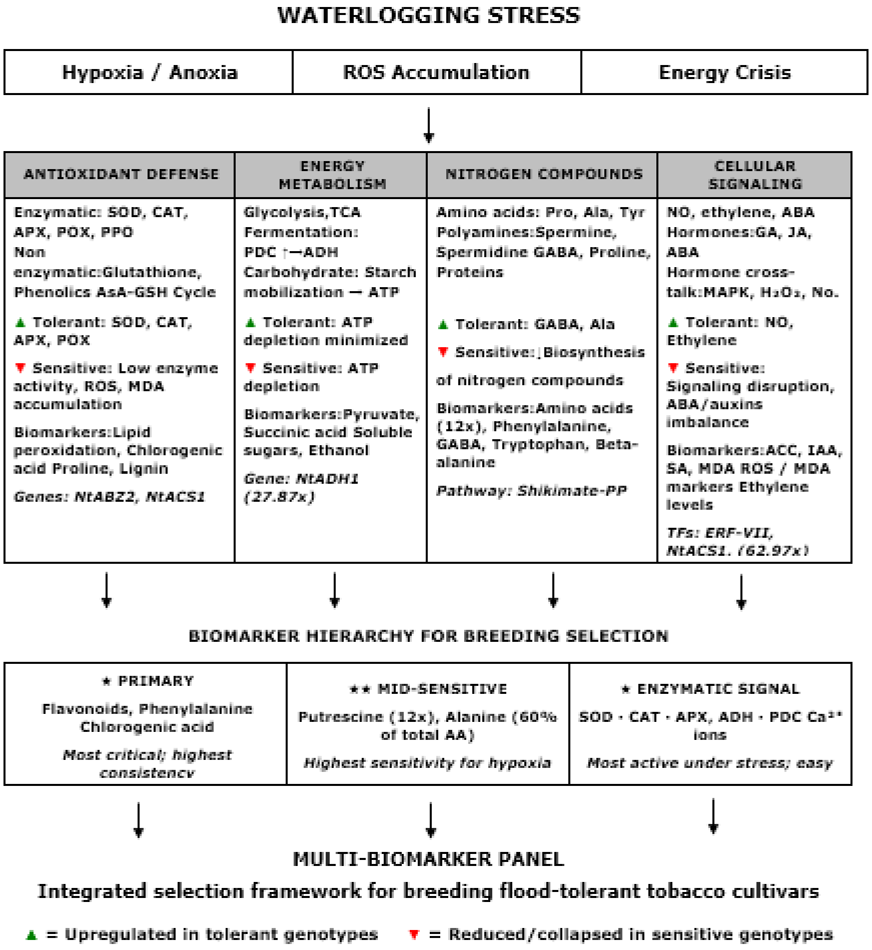

Figure 2. Conceptual model illustrating the integrated metabolic network and biomarker hierarchy in tobacco (*Nicotiana* spp.) under waterlogging stress.

overaccumulation of proline will create an increase in ROS and MDA production through the pyrroline-5-carboxylate pathway, which will decrease their capacity to handle stress, according to Szabados and Savouré (2010). The two functions of proline require scientists to use it as a biomarker for their research because it provides better results than the single-testing methods described in this study.

The integrated biomarker framework in Figure 2 demonstrates four primary metabolic defense mechanisms in tobacco under waterlogging stress: antioxidant defense, energy metabolism, nitrogen compounds, and cellular signaling. Tolerant genotypes exhibit significantly elevated enzymatic antioxidant activities (SOD, CAT, APX, and POX) that effectively scavenge ROS and minimize oxidative damage (Jaiswal and Srivastava, 2018; Gao *et al*., 2023). Energy metabolism adapts through enhanced fermentation with the *NtADH1* gene, showing 27.87-fold upregulation to maintain ATP production under anaerobic conditions (Pan *et al*., 2021; Gao *et al*., 2023). Nitrogen metabolism reveals differential patterns with GABA and alanine accumulation (60% of total amino acids), serving as osmotic protectants (Rocha *et al*., 2010; Lee *et al*., 2014). Cellular signaling demonstrates an *NtACS1* gene upregulation (62.97-fold) in tolerant genotypes, while sensitive cultivars suffer from hormonal imbalance and signaling disruption (Li *et al*., 2022; Bai *et al*., 2023).

The biomarker hierarchy in Figure 2 provides a practical breeding selection framework stratified by metabolite sensitivity and consistency. Primary biomarkers (flavonoids, phenylalanine, and chlorogenic acid) demonstrated the highest consistency for initial tolerance screening (Jaiswal and Srivastava, 2018). Midsensitive biomarkers, particularly putrescine (12-fold accumulation) and alanine (60% of total amino acids), exhibit the highest sensitivity to hypoxic conditions for stress intensity assessment (Rocha *et al*., 2010; Lee *et al*., 2014). Enzymatic signals (SOD, CAT, APX, ADH, PDC, and Ca^{2+}) enable real-time monitoring, though interpretation requires consideration of developmental stage and stress duration (Hasanuzzaman *et al*., 2020; Gao *et al*., 2023). This multi-biomarker panel integrates complementary metabolic pathways into a unified framework, substantially improving phenotypic selection accuracy for waterlogging tolerance in tobacco breeding programs (Phukan *et al*., 2018; Li *et al*., 2022).

Metabolic response of tolerant and susceptible genotypes

GABA accumulated predominantly in roots, mediating nicotine biosynthesis under hypoxia (Zhang *et al*., 2016). Alanine serves as an indicator of the metabolic transition from aerobic to anaerobic respiration (Lee *et al*., 2014), while beta-alanine contributes through the phenylpropanoid pathway (Oliva *et al*., 2021). The secondary metabolite profiles revealed distinct adaptive capabilities. Tolerant genotypes exhibited markedly increased biosynthesis of flavonoids (Nurhidayati *et al*., 2017; Pan *et al*., 2021) because their biosynthesis of flavonoids served to protect them from oxidative stress during hypoxia (Kuznetsov and Shevyakova, 2007). Chlorogenic acid functioned as an antioxidant and lignin precursor, which strengthened cell walls, according to Oliva *et al*. (2021), while anthocyanins showed higher accumulation in tolerant genotypes, according to Nurhidayati *et al*. (2017).

Tobacco genotypes display different responses to waterlogging because their metabolite profiles and content show different results (Table 3). Tolerant cultivars produce additional primary metabolites (specifically phenylalanine, tryptophan, and tyrosine) by basic biosyntheses. These primary metabolites are precursors of secondary metabolites through the shikimate-phenylpropanoid metabolic pathway. Tolerant genotypes of putrescine showed 12 times higher accumulation because they reached 12 times higher levels than the control group, while susceptible genotypes expressed only slight increases (Purnobasuki *et al*., 2020). All tobacco organs produce putrescine biosynthesis because ADC exists in chloroplasts and nuclei, according to the research of Kuznetsov and Shevyakova (2007), while spermidine and spermine increased to help stabilize membranes. These polyamines also function as osmotic protectants by maintaining cellular turgor pressure and water content through their hydrophilic nature, protecting macromolecules, and maintaining cell pH homeostasis at concentrations 1–2 orders lower than proline (Kuznetsov and Shevyakova, 2007).

The defense mechanisms function through three systems, which interconnect with each other, as shown in Figure 2. The anaerobic energy pathway, which uses PDC-ADH ethanol fermentation, produces ATP during hypoxic conditions. The antioxidant defense pathway protects cells from ROS-induced damage through its two defense mechanisms, which include enzymatic components (SOD, CAT, and APX) and non-enzymatic components (flavonoids, phenylpropanoids, and ascorbic acid). Third, stress-responsive gene networks get activated through the signaling and regulatory pathway, which uses Ca^{2+} ions, ethylene, and ABA. Purnobasuki *et al*. (2020) demonstrated that tolerant genotypes exhibit synchronized upregulation of NtADH1 (27.87-fold), NtABA2 (12.38-fold), and NtACS1 (60.97-fold), contrasting sharply with the diminished biosynthetic capacity and metabolic collapse in susceptible genotypes. The coordinated activation of these systems—rather than any

Table 3. Differences in the primary and secondary metabolites of tolerant and susceptible tobacco genotypes to waterlogging.

Group	Key metabolites / Enzymes	Tolerant genotype	Sensitive genotype	Main biological functions	Citations
Primary Metabolites	Putrescine, Alanine, GABA	Higher accumulation	Lower accumulation	Membrane stability, nitrogen homeostasis, stress signaling	Kuznetsov and Shevyakova (2007); Ju *et al*. (2014); Zhang *et al*. (2016)
	Phenylalanine, Tyrosine, Tryptophan	Enhanced biosynthesis	Reduced biosynthesis	Precursors for secondary metabolites and carbon recovery	Kuznetsov and Shevyakova (2007); Rivera-Contreras *et al*. (2016)
Secondary Metabolites	Flavonoids, Phenylpropanoids	High and stable content	Low content	Antioxidant defense, ROS scavenging, cell wall reinforcement	Pan *et al*. (2021)
	Anthocyanins, Chlorogenic acid	High accumulation	Low accumulation	Oxidative and photo-oxidative protection	Kuznetsov and Shevyakova (2007); Nurhidayati *et al*. (2017)
	Lignin	Increased accumulation	Reduced accumulation	Structural reinforcement and hypoxia adaptation	Nurhidayati *et al*. (2017)
Metabolic Enzymes	PDC, ADH	Increased activity	Decreased activity	Anaerobic ATP production and hypoxia tolerance	Gao *et al*. (2023)
Antioxidant Enzymes	SOD, CAT, APX	High enzymatic activity	Low enzymatic activity	ROS detoxification and oxidative protection	Gao *et al*. (2023)
Adaptive Responses	Oxidative and hypoxia tolerance	Strong adaptive response	High susceptibility	Maintenance of cellular integrity and survival	Kuznetsov and Shevyakova (2007)
	Membrane stability and recovery	Rapid post-stress recovery	Slow recovery	Restoration of metabolic balance after stress	Rivera-Contreras *et al*. (2016)
Gene Expression	NtADH1, NtABA2, NtACS1	Higher gene expression	Lower gene expression	Anaerobic metabolism, ABA signaling, and ethylene biosynthesis regulation	Purnobasuki *et al*. (2020)

single mechanism—distinguishes tolerant from susceptible genotypes and provides a conceptual basis for multi-target breeding strategies.

Tobacco metabolite tolerance to waterlogging

The systematic evaluation of candidate metabolic indicators across the reviewed literature shows a specific ranking that depends on three factors—citation frequency, functional relevance, and diagnostic sensitivity (Table 4). The assessment of metabolites showed antioxidant enzymes and energy-related metabolites and Ca^{2+} ions served as the most reliable biomarkers, which differentiated between tolerant and susceptible genotypes. The highest citation frequency for flavonoids (eight citations) demonstrates their

Table 4. Classification and functional roles of metabolic biomarkers in hypoxia and oxidative stress tolerance.

Biomarker	Category	Function/Role	Number of relevant citations	Citations
Putrescine	Polyamine	Energy metabolism regulation during hypoxia	4	Purnobasuki *et al*. (2020)
Phenylalanine	Aromatic amino acid	Precursor of phenylpropanoid pathway, biosynthesis of protective compounds, carbon source for recovery	6	Rivera-contreras *et al*. (2016); Oliva *et al.* (2021)
Alanine	Amino acid	Universal primary stress signal, hypoxia indicator, energy homeostasis	4	Ju *et al*. (2014)
Chlorogenic Acid	Phenylpropanoid	High antioxidant activity, lignin precursor, oxidative protection	5	Pan *et al*. (2021)
Flavonoids	Secondary metabolite	ROS scavenger, oxidative protection, antioxidant activity	8	Nurhidayati *et al*. (2017); Pan *et al*. (2021)
Anthocyanins	Flavonoid	Direct antioxidant protection, ROS reduction	4	Nurhidayati *et al*. (2017); Pan *et al*. (2021)
GABA (Gamma-Aminobutyric Acid)	Amino acid	Primary signal for nicotine biosynthesis, ROS damage reduction	3	Zhang *et al*. (2016)
Beta-alanine	Amino acid	Resistance response through phenylpropanoid pathway	2	Oliva *et al.* (2021)
Tryptophan	Aromatic amino acid	Increased biosynthesis, carbon source for recovery	3	Lee *et al*. (2014); Rivera-contreras *et al*. (2016)

recognized functions as multipurpose ROS scavengers, which protect against oxidative damage during hypoxic conditions (Kuznetsov and Shevyakova, 2007; Nurhidayati *et al*., 2017). The essential precursor of the shikimate-phenylpropanoid pathway, which serves as the major adaptive response to waterlogging, has successful identification through six citations of phenylalanine (Rivera-Contreras *et al*., 2016). Chlorogenic acid (five citations) functions as both an antioxidant and a lignin precursor, aiding in building cell walls during stress situations (Oliva *et al*., 2021). The research team identified beta-alanine and tryptophan as potential research subjects needing more investigation, although these two compounds receive a lower citation frequency (Figure 3).

Putrescine showed the highest capacity to diagnose hypoxia because it worked better than all other primary metabolites that respond to hypoxia. The tolerant genotypes reached 12 times higher putrescine levels than their control counterparts, while the susceptible genotypes provided only small increases, according to Purnobasuki *et al*. (2020). Alanine served as a reliable marker of the metabolic transition from aerobic to anaerobic respiration (Lee *et al*., 2014). GABA accumulated predominantly in roots, which processed nicotine production under oxygen deprivation, according to Zhang *et al*. (2016). The research activity on these metabolites shows an inverse exponential relationship because scientists study primary indicators more thoroughly, while they examine less common metabolites that contain unique tolerance characteristics.

The application of individual indicators requires assessment because their reliability varies across different levels of assessment. ADH activity, which responds to hypoxia, also shows activation during drought and cold stress conditions, leading to a decreased diagnostic accuracy (Pan *et al*., 2021). Certain species show that proline overaccumulation creates stress sensitivity instead of offering

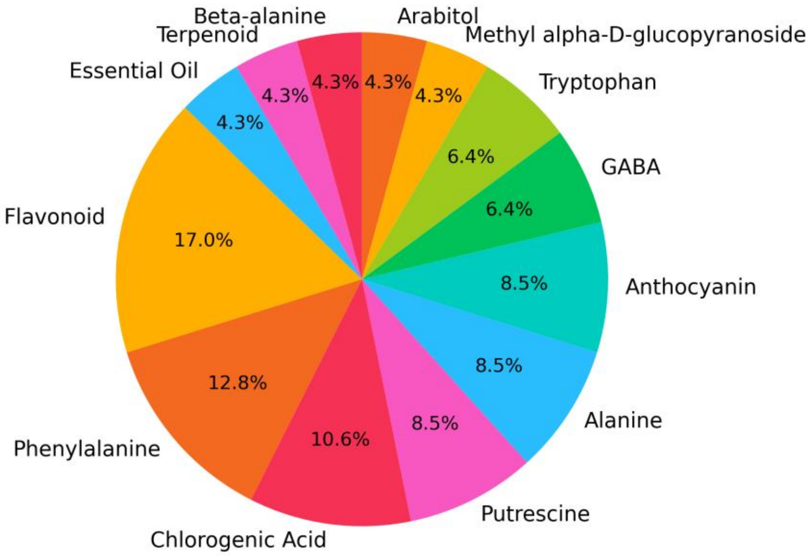

Figure 3. Relative importance of metabolic biomarkers based on citation frequency.

protective advantages (Szabados and Savouré, 2010). Antioxidant enzyme assays show higher levels in tolerant genotypes, but their measurement needs destructive spectrophotometric sampling methods, which are unable to support extensive germplasm testing. The study requirements demand a multi-indicator panel approach that uses different functional categories of metabolites to create superior predictive results through its combination of flavonoids, putrescine, and alanine versus using a single-indicator method (Figure 2). The panel establishes a strong basis for effective waterlogging-tolerant tobacco cultivar screening and selection through its combination with enzymatic activity assessments.

Breeding programs that use metabolite-based selection for their practical work need to examine the operational restrictions that exist in their operations. The heritability of metabolite traits, which include flavonoid accumulation and enzyme activities, depends on genotype × environment (G×E) interactions because identical genotypes produce different metabolite profiles under various field conditions. The analytical expenses for metabolomics platforms, using GC-MS and LC-MS technology, exceed the costs of traditional agro-morphological selection methods because destructive sampling techniques restrict sample processing capacity. The proposed multi-indicator panel should function as a secondary validation tool for promising genotypes identified through primary phenotypic screening because it does not serve as the main method for large-scale evaluation. Research should focus on creating inexpensive proxy measurements and non-destructive assessment techniques that can be effective in existing breeding pipelines while testing their suitability across various genetic backgrounds and production systems in different field environments.

Integration of transcriptomic and metabolomic perspectives in *Nicotiana*

The research establishes that transcriptomic and metabolomic data together provide an effective method to study the molecular mechanisms enabling tobacco plants to survive waterlogging. The gene expression analysis demonstrated the tolerant tobacco plants showed high NtADH1 expression, which reached 27.87 times the normal level, and they also showed high NtABA2 expression, reaching 12.38 times the normal level. Likewise, they showed high NtACS1 expression, which indicated 60.97 times the normal level at Marakot after they experienced periodic waterlogging, according to the research of Purnobasuki and his team. They showed the ADH enzyme activity increased together with the ethylene-driven metabolic changes described in this article. The multi-omics approach enables researchers to find regulatory networks controlling tolerance mechanisms while they create accurate molecular markers through the matching process between genes and metabolites. Omics-based research, involving multiple *Nicotiana* genotypes, will help researchers identify candidate genes and metabolic markers they would need to use for developing flood-tolerant breeding markers to create flood-tolerant cultivars.

Research gaps and future directions

The existing knowledge gaps need research to support tobacco breeding programs, which aim to develop varieties that can survive waterlogging conditions. The reviewed studies mainly used controlled short-term waterlogging experiments, yet researchers must validate biomarkers through testing in actual field environments involving changing temperatures and different soil types and living organisms, which exist in various tobacco-producing areas. The presented research only included specific genotypes, which makes it necessary to test all available *Nicotiana* germplasm to verify the discovered markers. The research should investigate how epigenetic changes and stress memory function together to create long-term tobacco metabolic adaptation. Research is also essential in investigating how multiple abiotic environmental stressors exist.

CONCLUSIONS

The review article provides evidence that tobacco genotypes have metabolic variations that work in an integrated way through different mechanisms, which include antioxidant defense, energy metabolism, nitrogen compounds, and cell signaling pathways. Tolerant tobacco genotypes exhibited higher levels of antioxidant and fermentation enzymes, energy reserves, and ATP than sensitive ones. This metabolic profile represents a clear distinction in flood resistance characteristics. Among the biomarkers, antioxidant enzymes, energy-related metabolites, and Ca^{2+} ions were recognizably better as the primary ones and considerably the most valuable criteria for selection in breeding programs aimed at developing flood-tolerant tobacco genotypes. This review provides the first systematic, tobacco-specific synthesis of metabolic responses to waterlogging stress. It critically evaluates the reliability of proposed biomarkers and advances an integrated multi-biomarker framework combining flavonoids, putrescine, alanine, and enzymatic indicators to enhance selection accuracy in breeding programs. Future efforts should focus on validating these biomarkers across diverse genotypes and field environments, integrating transcriptomic and metabolomic approaches for comprehensive pathway elucidation, and assessing genotype-by-environment interactions to support the development of climate-resilient tobacco cultivars.

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