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Breeding Strategies and Biotechnological Approaches to Reduce Nitrate Levels in Vegetables: A Comprehensive Review

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Nitrate is a natural chemical compound found in soil and water, which plants absorb and convert into essential nutrients. Elevated concentrations of nitrate in vegetables can pose health risks, particularly when consumed in substantial amounts. Green leafy vegetables, while crucial for human nutrition, unfortunately belong to the category of foods that have the highest impact on nitrate absorption in living organisms. Overuse of nitrogen fertiliser can lead to the accumulation of high levels of nitrate in these plants. Therefore, it is crucial to make efforts to reduce the nitrate levels in leafy vegetables and restrict their use by humans. Plant breeders employ several techniques, such as traditional breeding and genetic modification, to selectively produce plants with lower nitrate levels. The process frequently involves intentionally choosing and crossbreeding plants that naturally have lower levels of nitrates, so transmitting this trait to their offspring. This method is frequently conducted over multiple generations to ensure stable and uniform results. In addition, modern biotechnological techniques, such as genetic engineering or gene editing, can be employed to directly modify the genes responsible for nitrate metabolism in plants. Advanced techniques such as integrated multi-omics, RNAi, gene editing, and genomics-assisted breeding are applied to create crops with fewer undesirable traits and to develop new strategies for regulating these traits in crop improvement projects.

Keywords: Nitrates; vegetables; health hazards; breeding approaches.

1. INTRODUCTION

Vegetables are grown in many parts of the globe and are a significant source of minerals, phytochemicals, dietary fiber, and vitamins such as C, A, B1, B6, B9, and E [1]. There are several categories of vegetables. They might potentially be categorized as fruit, seeds, fruits, stalks, leaves, or edible roots. According to Robinson [2], every group has a distinct impact on the diet. The diet is enhanced by the presence of fiber, vitamins, and minerals that are present in veggies. Minerals are naturally occurring inorganic substances with a defined chemical composition and a structured atomic arrangement. Vegetables are a kind of plant that is rich in minerals and plays a significant role in meeting the Recommended Dietary Allowance (RDA) for these vital elements. However, certain vegetables include nutrients that might potentially decrease the bioavailability of such nutrients. In 2007, scientists Fowomola [3] conducted study to investigate and describe the anti-nutritional properties of plant fruits and seeds. The molecules or chemical compounds found in fruits and other food products that have the potential to harm people are referred to as anti-nutritional factors. Ugwu and Oranye [4] found that plant material had limited potential to retain nutrients such as minerals, vitamins, and proteins. Vegetables are a crucial component of the human diet and are believed to be a significant contributor to the buildup of nitrates [5-7], making about 72%-94% of the total nitrate intake by individuals [8]. While nitrate alone is not very hazardous, it has the potential to undergo internal conversion into nitrite, which may react with amines and amides to produce N-nitroso compounds [9]. Choi et al. [10] found that many drugs are associated with an increased risk of illnesses. Excessive nitrate may competitively hinder the absorption of iodide via the sodium iodide symporter [11]. As a result, the presence of nitrate in vegetables has garnered more attention. Extensive research has been conducted in recent years to minimize the occurrence of nitrate in vegetables. Nevertheless, the accumulation of nitrates is a multifaceted trait, and both internal and external factors have an effect on the nitrate content of plants [12]. Moreover, the nitrate concentration varies considerably across different plant species and cultivars [13]. Therefore, choosing genotypes that have lower nitrate accumulation might greatly reduce the nitrate intake of people from vegetables.

2. NITRATE ACCUMULATION IN VEGETABLES

Vegetables may have beneficial effects on health, however it is crucial to consider their levels of nitrates and nitrites. The main growth regulators used in most field crops are nitrate and ammonium, which are forms of mineralized nitrogen. In order to enhance agricultural productivity, farmers may use nitrogen-rich manure and fertilizers. A significant quantity of nitrate may build in several green leafy vegetables. Variables such as season, light, temperature, growing conditions, fertilizer administration, and crop storage have an impact on concentrations, as stated by Dich et al. [14]. Several variables, such as the kind of plant, various varieties of the same plant species, and even variations in genetic makeup with varied ploidy levels, influence the nitrate levels in plants. The genetic regulation of shoot nitrate content is determined by many genes, also known as quantitative trait loci (QTLs) [15].

3. NITRATE DISTRIBUTION WITHIN THE PLANT

The Brassicaceae (rocket, radish, mustard), Chenopodiaceae (beetroot, Swiss chard, spinach), and Amarantaceae families are known for producing vegetables that accumulate nitrates. However, high nitrate levels can also be found in certain species of the Asteraceae (lettuce) and Apiaceae (celery, parsley) groups (Table 1). The varying amounts of nitrate absorption and transport throughout the plant, together with the diverse sites of nitrate reductase activity, are all associated with distinct abilities to accumulate nitrate [16]. Nitrate levels inside a plant exhibit fluctuations. Undoubtedly, the below enumeration of vegetable organs may be arranged in descending order based on their nitrate content [17]. The sequence of these items, from highest to lowest, is as follows: petiole, leaf, stem, root, inflorescence, tuber, bulb, fruit, and seed. The nitrate levels in root vegetables were higher compared to fruit and vegetables, although leafy vegetables exhibited even higher quantities than the other types [18].

3.1 Nitrate Toxicity

The presence of excessive levels of nitrates in vegetables is a worldwide concern. Various regions, such as Mainland China and many European countries have shown very elevated levels of nitrate in vegetables, namely leafy greens (exceeding 5,000 mg/kg) [19]. The nitrate levels in vegetables and water may be higher now compared to the past, mostly because of the increased use of animal dung and synthetic nitrogen fertilizers in intensive farming practices. Methaemoglobinemia is the most immediate hazardous consequence of nitrite poisoning, resulting from the reaction between nitric oxide (NO_2) and oxyhemoglobin (Fe2+) to form methemoglobin (Fe3+) and nitrate (NO_3). The blood is the specific organ being targeted. Haemoglobin (Hb), a ferrous molecule present in blood, is responsible for the transportation of oxygen. Methaemoglobin (metHb) is formed from haemoglobin (Hb) in the presence of nitrite and is unable to carry oxygen. The typical level of metHb in people is below 2%, and in newborns under three months, it is below 3%. The low level of metHb is maintained by a series of enzymatic mechanisms that consistently convert metHb back to Hb [20]. Cyanosis, characterized by the appearance of blue skin and lips, often becomes noticeable when methemoglobin (metHb) levels reach 10% or more of normal hemoglobin (Hb) concentrations.

3.2 Acceptable Daily Intake

The Joint Expert Committee of the Food and Agriculture (JECFA) Organization of the United Nations/World Health Organization (WHO) provides a definition for the term ADI, which applies to pollutants (such as pesticides, herbicides, and fertilizers) as well as compounds intentionally added to food [21]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food (SCF) of the European Commission have set an Acceptable Daily Intake (ADI) for NO3 ranging from 0 to 3.7mg per kilogram of bodyweight. Mensinga et al. [22] said that the US Environmental Protection Agency (EPA) has set the Reference Dose (RfD) for nitrate at 1.6 mg of nitrate nitrogen per kilogram of body weight (bw) per day, which is approximately equivalent to 7.0 mg of NO_3 per kilogram.

4. BREEDING STRATEGIES FOR LOW NITRATE CONTENT

One important breeding approach that helps to improve the quality of product is reducing the amount of anti-nutrients in crops [23,24]. The process of decreasing nitrate levels in crops is an essential breeding technique that significantly contributes to improving the quality of the harvest. Several conventional techniques and technologies may be used to decrease the concentrations of these anti-nutrient compounds. Various processing processes such as fermentation, germination, debranning, autoclaving, and soaking are used to decrease the levels of anti-nutrients in foods [25-29]. Through the utilization of diverse techniques, either alone or in conjunction, it is feasible to diminish the concentration of anti-nutrients in food sources [30]. Various breeding procedures, including selection, mutation, backcrossing, hybridization, and population enhancement, have been used using both natural and induced genetic resources [31]. The effort to decrease the presence of substances that inhibit nutrient absorption in crops began in the early 1960s with the cultivation of cotton plants that lacked glands producing these substances. In recent times, gene silencing and editing methods have been used to create significant crop varieties with reduced levels of anti-nutrients. The process of reducing anti-nutrient accumulation in plants by conventional breeding started by identifying germplasm accessions with lower levels of anti-nutrients [23]. Below are many traditional breeding techniques used to decrease nitrate concentrations in vegetables:

Plant breeders seek naturally existing vegetable types with reduced nitrate levels. Through the process of finding and choosing these specific kinds that have low levels of nitrate, scientists may develop new plant lines that have a decreased amount of nitrate. The nitrate levels observed in the four leafy vegetables analyzed align with the categorization outlined by Colla et al. [32]. According to their classification, lettuce has a moderate nitrate content, spinach has a high nitrate content, watercress has a very high nitrate content, and rocket has an exceptionally high nitrate content. Observing the selection of pulse germplasm for reduced enzyme inhibitors was shown to be an effective method for identifying prospective donors with decreased inhibitor levels. Two soybean lines, PI 157-440 and PI 196-168, were found to have no Kunitz inhibitor [33]. The regulation of these inhibitors was determined by a recessive gene (*tj)* which was subsequently included into a high-quality cultivar [34]. The abbreviation "vc" was awarded for its role in diminishing the activity of enzyme inhibitors [35-38].

Cross-breeding is the deliberate process of marrying two distinct plant kinds that possess desired characteristics [39-42]. Breeders may get offspring with intermediate nitrate levels by crossing high-nitrate types with low-nitrate ones. By engaging in a series of generations of cross-breeding and selection, they may ultimately get vegetable varieties with notably decreased nitrate content. In a study conducted by Nieuwhof [43], the potential for decreasing the nitrate levels in radish roots through breeding was examined. Different radish plants with varying nitrate content were chosen from four populations of the diploid cultivar Robijn (two populations with low nitrate content, one with intermediate nitrate content, and one with high nitrate content), as well as from two tetraploid cultivars, 'Boy' and 'Oscar', which had low nitrate levels. Significant disparities in nitrate content were observed between the parent populations, with certain populations having much higher or lower nitrate concentrations compared to their respective parents. In addition, he emerged from crosses involving cytoplasmic-genetic male sterile plants. There was no correlation seen between cytoplasmic-genetic male sterility and nitrate content. This suggests that hybrid cultivars with low nitrate levels may also be chosen.

Mutagenesis is a method in which plant seeds or tissues are subjected to mutagenic agents, such as chemicals or radiation, in order to induce genetic changes. These genetic alterations may result in modifications to the process of nitrate metabolism. Subsequently, breeders evaluate the mutant plants to identify individuals with low-nitrate qualities and choose those exhibiting the required characteristics for further breeding. The UV approach is a fast and uncomplicated technique that might be useful for determining the overall amount of vicine in different cultivars and in faba bean products intended for human consumption [44]. Through the use of induced

Table 1. Nitrite and nitrate contents of edible components in vegetables [9]

Vegetables	Nitrite (mg/100 g fresh weight)	Nitrate (mg/100 g fresh weigh)
Carrot	0.002–0.023	92–195
Mustard leaf	0.012–0.064	70–95
Lettuce	0.008–0.215	12.3–267.8
Spinach	0–0.073	23.9–387.2
Chinese cabbage	0–0.065	42.9–161.0
Bokchoy	0.009–0.242	102.3–309.8
Cabbage	0–0.041	25.9–125.0
Cole	0.364–0.535	76.6–136.5
Wax gourd	0.001–0.006	35.8–68.0
Cucumber	0–0.011	1.2–14.3
Eggplant	0.007–0.049	25.0–42.4

mutations, three mutants were generated in the M_5 generation that exhibited reduced levels of lectin and a normal germination rate, as reported by George et al. [45].

Genetic analysis is used in MAS to identify particular genetic markers that are connected with features related to low-nitrate. These markers function as indications for the existence of the target characteristic without requiring time-consuming phenotypic screening. This enables breeders to more effectively choose plants with a reduced amount of nitrates. Molecular markers are diagnostic instruments that may be used to identify the existence of certain traits that have economic significance. Marker aided backcrossing (MABC) is a very useful technique in breeding that is widely used in several research studies aimed at identifying specific genomic areas of interest [46].

Backcrossing is a method in which the hybrid offspring produced from a cross between two kinds are then crossed with one of the original parent varieties. This process is iterated throughout several generations to preserve the favorable characteristics of one parent while diminishing the influence of the other parent's unfavorable qualities, such as elevated nitrate levels. Reinink [47] conducted research on lettuce and discovered significant genetic variation in nitrate levels. Genetic studies have shown that the nitrate concentration in lettuce is a quantitative trait, mostly controlled by dominant genes that result in low nitrate content. By crossing low nitrate cultivars, it has been shown that it is feasible to achieve a further decrease in nitrate content via the accumulation of genes associated with low nitrate levels.

5. ADVANCED BREEDING APPROACHES

Modifying anti-nutritional characteristics to improve the availability of nutrients is a significant issue in crops, since these characteristics must be reduced in a manner that prevents detrimental impacts on crop output. The mitigation of these characteristics in crops has been effectively executed for significant anti-nutrients, and the diverse techniques for modifying their content are elucidated [48]. RNAi technology is a very effective method for suppressing gene activity, which may be used to enhance the expression of certain factors in crops. This technique has been successfully employed to target genes that are responsible for producing these components in plants. Gossypol, a plant phytochemical, significantly contributes to host plant resistance and is not essential for human nutrition. Thus, the d-cadinene synthase gene has been silenced to create ultra-low-gossypol cotton. The gene knockdown resulted in a decrease in the accumulation of gossypol in the seeds, leaves, and floral organs of transgenic cotton. The first iteration of transgenic cotton demonstrated comparable performance, in terms of both crop production and fiber quality, to that of steady expression. According to Palle et al. [49], the transgenic cotton showed greater oil content compared to the control. In a recent study by Rathore et al. [50], targeted RNA interference (RNAi) was used to disrupt the d-cadinene gossypol gene in seeds of the TAM66274 cultivar. This resulted in a significant reduction in oil content by around 97%. Furthermore, the cultivar successfully passed food safety assessments undertaken by the Food and Agricultural Organization of the USA (FAO).

Valentine et al. [51] used metabolite engineering to manipulate the content of raffinose in soybean. The inhibition of the cucumber stachyose synthase gene (*CsSTS*) by RNAi-induced silencing has a notable effect on the process of phloem loading, the metabolism of carbohydrates, and the ability to tolerate low temperatures [52]. A recent study by Le et al. [53] demonstrated the efficacy of a sophisticated gene editing method that used two guide RNAs to disable the *GmGoLS1A* and *GmGoLS1B* genes responsible for galactinol synthase in soybean. This intervention led to a significant drop in raffinose levels, reducing it from 64.70 mg/g to 41.95 mg/g, or a 35% reduction. After the successful use of RNA interference (RNAi) in IPK to decrease phytic acid levels, the CRISPR-Cas9 technique has recently been employed on a comparable gene in soybean called *GmIPK* to modify the concentrations of phytate in soybean. The objective of this work was to establish a standardized method for transforming transgenic soybean lines with modified *GmIPK2*. This highlights the need to prioritize the development of more bioinformatics tools and research on transient expression in order to enhance the quality of soybean meal using CRISPR technology in the future [54]. Biotechnological breeding approaches seek to augment favorable characteristics in plants, such as reducing the nitrate concentration in vegetables. Elevated nitrate concentrations in vegetables may provide a health risk, especially for certain vulnerable groups. Biotechnological solutions provide focused and accurate means to accomplish this objective. Marker-assisted selection (MAS), CRISPR-Cas9 gene editing, RNA interference (RNAi), and transgenic methods. Metabolic engineering and mutagenesis, which are biotechnological breeding approaches, show great potential. However, they need to undergo thorough testing and get regulatory permission to assure their safety for both consumption and the environment.

6. FUTURE PROSPECTS

Advanced genomic techniques, including as CRISPR/Cas9 and other gene-editing tools, will be crucial in the development of crop cultivars that have reduced nitrate composition. These technologies enable accurate alterations in the genes involved for nitrate absorption and assimilation, hence improving the efficiency of these processes. Marker-assisted selection (MAS) will accelerate the progress of developing low-nitrate cultivars by identifying and utilizing genetic markers that are associated with desired features. In addition, transcriptome and metabolomic investigations will offer a more comprehensive understanding of the molecular mechanisms that regulate nitrate metabolism, enabling particular approaches. Biotechnological advancements will also investigate the use of advantageous microorganisms, such as nitrate-reducing bacteria, to mutually decrease nitrate levels in plants. In addition, combining these sophisticated procedures with conventional breeding methods will ensure the creation of resilient, high-productivity, and low-nitrate vegetable cultivars. The collective endeavors in these domains will greatly contribute to the promotion of sustainable agriculture methods, enhancing both the safety and quality of food, while simultaneously mitigating the adverse environmental consequences of excessive nitrate utilization.

7. CONCLUSION

Plants absorb nitrate from soil and water to make nutrients. When consumed in large amounts, vegetable nitrate levels may be harmful. Despite their nutritional value, green leafy vegetables are the meals that significantly increase nitrate absorption. Excess nitrogen fertilizer may lead these vegetables to build high amounts of nitrate, which may be harmful if eaten. Thus, green vegetables should have fewer nitrates and people should consume less. Plant breeders selectively breed for low nitrate content using traditional breeding and genetic modification. Plants with low nitrate levels are selected and interbred to pass on this trait to their offspring. This technique is repeated over generations to ensure consistency. Modern biotechnological approaches like genetic engineering and gene editing may directly affect plant nitrate metabolism genes. By identifying and altering critical genes involved in nitrate uptake, breeding to eliminate anti-nutrition factors may compromise crop output and seed size. In agricultural improvement programmes, integrated multi-omics, RNAi, gene editing, and genomics-assisted breeding are employed to generate crops with fewer undesirable features and innovative management methods.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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