

Perspective

New Breeding Techniques for Greenhouse Gas (GHG) Mitigation: Plants May Express Nitrous Oxide Reductase

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Abstract: Nitrous oxide (N₂O) is a potent greenhouse gas (GHG). Although it comprises only 0.03% of total GHGs produced, N₂O makes a marked contribution to global warming. Much of the N₂O in the atmosphere issues from incomplete bacterial denitrification processes acting on high levels of nitrogen (N) in the soil due to fertilizer usage. Using less fertilizer is the obvious solution for denitrification mitigation, but there is a significant drawback (especially where not enough N is available for the crop via N deposition, irrigation water, mineral soil N, or mineralization of organic matter): some crops require high-N fertilizer to produce the yields necessary to help feed the world's increasing population. Alternatives for denitrification have considerable caveats. The long-standing promise of genetic modification for N fixation may be expanded now to enhance dissimilatory denitrification via genetic engineering. Biotechnology may solve what is thought to be a pivotal environmental challenge of the 21st century, reducing GHGs. Current approaches towards N₂O mitigation are examined here, revealing an innovative solution for producing staple crops that can 'crack' N₂O. The transfer of the bacterial nitrous oxide reductase gene (*nosZ*) into plants may herald the development of plants that express the nitrous oxide reductase enzyme (N₂OR). This tactic would parallel the precedents of using the molecular toolkit innately offered by the soil microflora to reduce the environmental footprint of agriculture.

Keywords: radiative warming; atmospheric phytoremediation; N₂O; nitrous oxide reductase; N₂OR; *nosZ*; fertilizer; crop breeding; transgenic; GHG

1. Introduction—Nitrous Oxide Continues to Bloom Unabated

Atmospheric nitrogen (N) deposition is a pressing matter for climate change scientists concerned with the increasing danger that nitrous oxide (N₂O), a noxious greenhouse gas (GHG), poses. Reactive nitrogen (Nr)—ammonia (NH₃), nitrogen oxides (NO_x), nitrates (NO₃[−]), and N₂O—enters the biosphere from its original form of atmospheric N as at least three derivatives: gas, dry deposit, and precipitation (wet deposition) [1,2]. The sources of N₂O are largely anthropogenic [3]. Many crops must receive N-based fertilizer to reach yield targets, which is supplied by inorganic fertilizers and animal manure [4]. In an effort to boost the yield in crop staples like wheat, corn, and soybeans, farmers apply N fertilizers at rates and times that are not always properly synchronized with crop demand [5]. While crops thrive when fertilized, experimental analysis has demonstrated that up to

40% of fertilizer N can be lost via leaching [6,7]. Other routes of N loss include soil erosion, NH_3 volatilization and oxidation, and bacterial/fungal denitrification [8], although N losses through NH_3 volatilization are higher than those via N leaching [9]. Around 62% of total global N_2O issues from natural and agricultural soils, and the bulk of this production, mainly results from the processes of bacterial nitrification and denitrification [10].

Nr compounds enter the atmosphere through biological processes, but the invention of the Haber-Bosch process in 1908 was a critical moment for the sudden increase in Nr and GHG production globally [11]. This process of artificial N-fixation allowed for the large-scale reduction of N_2 to NH_3 , producing massive amounts of synthetic N-based fertilizers that supported dramatic increases in high-yield farming [12]. This process now accounts for 80% of anthropogenic N-fixation (the remaining 20% resulting from combustion [13], with anthropogenic N-fixation in turn accounting for 60% of global N-fixation [14]). Haber-Bosch remains the industry standard synthetic N fertilizer today and as a result, has contributed to the ~2% increase in atmospheric levels of N_2O [15,16]. This effect is also magnified by the global emissions of N_2O produced by fossil fuel combustion [17] and the natural ability of legumes to fix N through symbiotic relationships with soil bacteria [18].

N_2O is the third most prevalent GHG, behind carbon dioxide (CO_2) and methane (CH_4) [19]. The concentration of this gas in the atmosphere has been steadily increasing since the early 1900s (Figure 1), and it is 265 times more radiative than CO_2 [19]. N_2O also has an atmospheric lifetime of 121 years; by comparison, CH_4 has an atmospheric lifetime of only 12 years, but CO_2 also has a long half-life and can take anywhere from 20–200 years to be absorbed by the ocean [19], compounding the ‘greenhouse gas’ effect. Since chlorofluorocarbons (CFCs) were banned in 1989, N_2O has become the leading cause of ozone layer depletion [20].

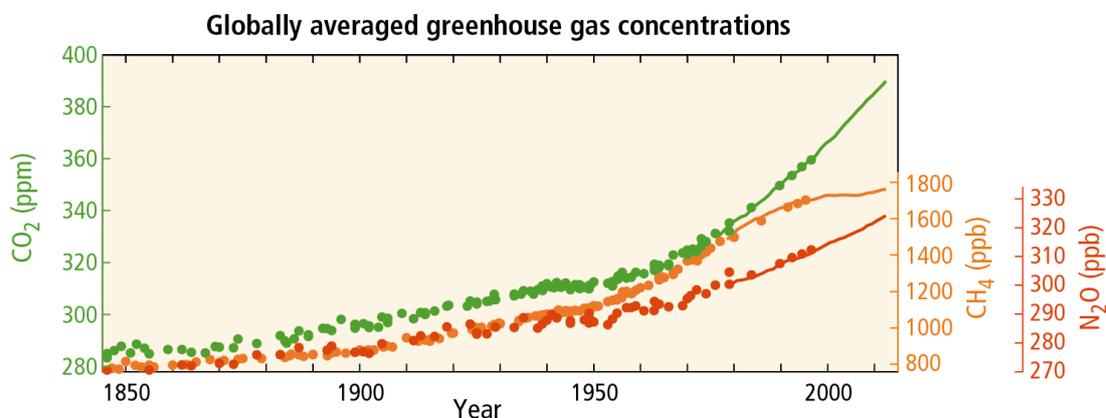


Figure 1. GHG levels since 1850. The green line represents the increase in CO_2 concentration since 1850; the orange line represents the increase in CH_4 concentration since 1850; lastly, the red line represents the increase in N_2O since 1850 [19].

N_2O emission results from the coupled oxidation and reduction of N performed by heterotrophic [21] (and some autotrophic) soil proteobacteria: (1) the nitrification pathway is catalyzed by autotrophs (*Nitrosomonas* spp. and other genera [22]) and also heterotrophs, and involves the oxidation of NH_3 /ammonium (NH_4^+) to nitrite (NO_2^-) [23] and nitric oxide (NO) [24]), which is followed by the oxidation of NO_2^- to NO_3^- by *Nitrobacter* spp. [25]; and (2) the denitrification pathway, whereby NO_3^- is reduced to N_2O and ultimately inert N_2 gas [26]. As many as a third of soil bacterial species [27] lack the *nosZ* gene that reduces N_2O to inert N_2 [28], which leads to a sizeable amount of incomplete denitrification reactions and the subsequent buildup of N_2O since it is an obligate intermediate [29]. This N_2O diffuses out of the soil and into the atmosphere, contributing to the greenhouse effect, contaminating water, and leading to serious human health implications [30,31].

2. Combating GHGs: Current N₂O Mitigation Strategies and Limitations

Demands for crop-borne food must be met, and so researchers must address the hazards of N-based fertilizers [32]. There are multiple N₂O mitigation strategies either currently in commercial use or in development (summarized in Table 1).

Table 1. Summary of current N₂O mitigation strategies.

Strategy	Mechanism of Action	Pros	Cons
(1) Conservation tillage and crop rotation [33]	Tillage, rotation of N-fixing crops, cover cropping [33]	Prevent NH ₃ volatilization and eventual N ₂ O emissions [34,35]	Unreliable N ₂ O mitigation [36,37]. Yield reduction [38]. Not effective at scrubbing N ₂ O from the air
(2) Best management practices (BMPs) [39]	Correct source, placement, time, and rate of fertilization [40]. Proper irrigation (fertigation) [41]	Proven to reduce N ₂ O emissions [41] and other N losses [42]	Technical constraints [43]
(3) EENFs [44]	Multiple types: stable, short-release (SRFs), and constant-release (CRFs); rely on enrichment of chemical inhibitors or coated N-compounds that are released into the soil over a period of time [45]; urease inhibitors (UIs) [46]	Proven to reduce N ₂ O emissions [47,48]	Inconsistent yields from year to year [48]. More expensive than standard N fertilizers [49]. Long lifetime of N-compounds in soil can lead to NH ₃ volatilization [50,51]. Not effective at scrubbing N ₂ O from the air
(4) Synthetic N ₂ O mitigators	SNIs suppress activity of nitrifying bacteria in the soil [52]. SDIs operate by unknown mechanism [44,53,54]	SNIs and SDIs reduce N ₂ O emissions [52,54]	Effectiveness depends on environmental conditions, prefer low temperature and sandy soils [55]. Not effective at scrubbing N ₂ O from the air
(5) Biological N ₂ O mitigators	BNIs suppress activity of nitrifying bacteria in the soil by releasing compounds that inhibit NH ₃ -oxidizing pathways [56]. BDIs inhibit nitrate reductase to inhibit N ₂ O production [57]	BNIs demonstrated to reduce N ₂ O emission [56]; BDIs inhibit denitrification and can conceivably mitigate N ₂ O emissions [57]	BNI-exuding plants must be grown in rotation with other crops [58]. Little work done on BDI-exuding plants [57]. Not effective at scrubbing N ₂ O from the air
(6) Microbial bioremediation	Proper water table management to facilitate growth of rhizobia [59]; inoculation of plant roots with genetically modified N ₂ O-cracking rhizobia [60,61]	Enables plants to degrade contaminants in the soil; N ₂ O-cracking rhizobia demonstrated to reduce N ₂ O emissions [60,61]	Most effective on crops that naturally cultivate a rhizosphere of N ₂ O-reducing [62] microorganisms, i.e., soybean [63]. Not effective at scrubbing N ₂ O from the air
(7) Rhizosecretion	Transformation of amenable crops to express recombinant bacterial proteins that reduce N ₂ O [64]	Plants that secrete N ₂ O-cracking enzyme could target N ₂ O in soil [64]	Plant transformation is a time-consuming process [65]. Bacterial proteins may not function efficiently in heterologous hosts [66]. Not effective at scrubbing N ₂ O from the air
(8) Atmospheric phytoremediation	Transformation of amenable crops with genes expressing recombinant bacterial proteins that reduce N ₂ O [67]	Arm crops and other plant species to mop up N ₂ O in the atmosphere [67], including N ₂ O emitted by other non-agricultural sources	Plant transformation is a time-consuming process [65]. Bacterial genes may not function in a heterologous system [66]. Not yet experimentally validated via gas analysis

BDI, biological denitrification inhibitor; BNI, biological nitrification inhibitor; EENFs, enhanced efficiency nitrogen fertilizers; SDI, synthetic denitrification inhibitor; SNI, synthetic nitrification inhibitor.

- (1) **Conservation tillage and crop rotation.** Mechanical incorporation (tillage) of N-based fertilizer into the soil may also be effective [68], but this is affected by many other parameters, such as the method of N application (i.e., broadcast vs surface urea ammonium nitrate). These techniques also result in a reduced yield [38]. Conservation tillage increases N₂O emissions compared with no-till and conventional tillage techniques using broadcast application, while tillage in general does not reduce N₂O emissions produced from surface urea ammonium nitrate-treated fields [69]. Other studies have shown that conservation tillage *reduces* N₂O emissions [70], underscoring the lack of reliability of this N management technique [36,37]. Crop rotation with N-acquisitive plant species can also reduce N₂O emissions following the application of high N-fertilizer treatment [33]; cover cropping can also control N₂O emissions, but the results are often variable and in some cases can increase N₂O emissions [71];
- (2) **Best management practices (BMP) [39].** Such nitrogen use efficiency techniques are myriad and involve simple steps such as proper fertilizer placement, timing of fertilizer application, the right type of N-compound, and so on. Others involve the proper incorporation of N-compounds into the soil so that they may be taken up by the plant more effectively and will be less likely to volatilize [72]. Fertigation, a technique involving careful irrigation of fields following the application of N fertilizer, is effective at mitigating N₂O emissions [41]. Such knowledge-based N management practices have been shown to be effective at both increasing crop yield and reducing immediate N₂O emissions [73], but some approaches may also increase N₂O production in the long term [55]. Their effectiveness also depends heavily on proper practices put in place by the farmers themselves, which requires proper training [43];
- (3) **Fertilizer management using enhanced efficiency nitrogen fertilizers (EENFs).** These fertilizer cocktails are concocted in such a way that they prevent the volatilization of NH₃ and inhibit nitrification/denitrification [46]. EENFs generally fall into one of three categories: (a) stabilized fertilizers, which contain nitrification and/or urease inhibitors; (b) slow-release fertilizers (SRFs), whereby the N source in the fertilizer is released over time from encapsulated granules, although the release rates can be variable; and (c) controlled-release fertilizers (CRFs), where the release rate is constant [45]. Urease inhibitors (UIs) are also a common EENF component. N-(*n*-butyl) thiophosphoric triamide (NBPT), phenylphosphorodiamidate (PPD), and hydroquinone are used worldwide and act by inhibiting the bacterial hydrolysis of urea into NH₃ in fertilizer [46,74,75]. UIs are typically used in conjunction with nitrification inhibitor (NIs) for maximum effectiveness [76,77], but NBPT alone can reduce N₂O emissions from N-treated soil [78]. There is controversy regarding the effectiveness of EENFs; while reductions in N₂O emissions from the soil have been recorded [47,48], recent studies have shown that crop yields are only marginally higher when EENFs are used in place of standard N fertilizers [79]. Those studies that demonstrated reduced N₂O emissions also reported inconsistent results from year to year [50]. Questionable effectiveness notwithstanding, EENFs are more expensive than conventional N-containing fertilizers and require special handling and storage [49,80], which are all features that make these fertilizers less attractive to farmers;
- (4) **Synthetic N₂O mitigators.** Synthetic nitrification inhibitors (SNIs) and UIs are both used in EENFs and can be applied to crops in conjunction with standard N fertilizer. NIs inhibit the activity of *Nitrosomonas* to block the nitrification of N in fertilizer (the oxidation of NH₃ to hydroxylamine via ammonia monooxygenase (AMO)) [23,52]. The efficacy of the inhibitors is also dependent on environmental conditions, as they are unstable; 3,4-dimethylpyrazole phosphate (DMPP), for example, exhibited reduced activity in hot, dry conditions [81]. The use of these inhibitors can also lead to less than desirable results: DMPP and 3-methylpyrazole 1,2,4-triazole (3MP + TZ) have been shown to increase N₂O emissions in vegetable crop systems, as the inhibitors promote the buildup of N in the fraction of the soil most available to bacteria during the breakdown of vegetative matter. Synthetic denitrification inhibitors (SDIs) suppress denitrification via unknown mechanisms [82], although some are known to inhibit the activity of fungal copper reductase [83].

- SDIs nitrapyrin [84], toluidine [54], and acetylene [44] all effectively mitigate N₂O emission, albeit with toxic side-effects [55], and they do not technically inhibit nitric oxide reductase;
- (5) **Biological N₂O mitigators.** This category is comprised of compounds produced by plants that inhibit enzymes in either the bacterial nitrification or denitrification pathway. The exploitation of such inhibiting root exudates is another intriguing approach towards N₂O mitigation [82]. Biological nitrification inhibitors (BNIs) are compounds that block the activity of NO₂⁻ producing enzymes. The roots of the tropical grass *Brachiaria humidicola* exude brachialactone, a compound that can mitigate N₂O emission from soil [85]. Attempts at developing BNI-producing cultivated wheat by crossing *Triticum aestivum* with BNI-producer *Leymus racemosus*, a wild wheat, have imparted some BNI activity, but also made the lines susceptible to rust infection [86]. The use of BNIs as an effective N₂O mitigator is also severely limited by the fact that the enactor of nitrification is a plant itself and cannot be applied to growing crops, although growing *B. humidicola* in rotation with maize saw a four-fold increase in yield [87]. Biological denitrification inhibitors (BDIs) are a relatively new discovery. Currently, the only example of such an inhibitor is the procyanidin produced by the invasive *Fallopia* spp. (Asian knotweed). This compound has been demonstrated to be an allosteric inhibitor of *Pseudomonas brassicacearum* nitrate reductase and while it does reduce denitrification in the soil, it has not yet been proven to mitigate N₂O levels [57];
 - (6) **Microbial bioremediation [88].** The success of N fertilizer management techniques and proper irrigation is largely due to the creation of a microsphere conducive to denitrifying bacteria flourishing [89]. Proper water table management techniques can promote the growth of N₂O-cracking bacteria in the soil and reduce N₂O emissions from the managed soil regions [59]. Another type of microbial bioremediation takes advantage of the ability of certain bacterial species to inhabit the root nodules of leguminous crops. Field peas [62], broad beans [90], and soybean [63] house bacteria (or rhizobia) that fix N and, unfortunately, also produce N₂O gas. While maintaining the rhizosphere, N₂O emissions can be mitigated by inoculating the roots of leguminous plants with rhizobia modified to express higher levels of a bacterial N₂O-cracking enzyme [60]. Genetically engineered strains of *Bradyrhizobium japonicum* have been used to inoculate the roots of soybean and reduced N₂O emissions [61]. Needless to say, this method is far more effective on crops that naturally cultivate a rhizosphere of N₂O-reducing microorganisms. It is also another technique that cannot target atmospheric N₂O;
 - (7) **Rhizosecretion.** This is a biotechnology-based approach, involving the transformation of amenable crop plants with genes expressing recombinant bacterial proteins that reduce N₂O by secreting N₂O-cracking enzymes [64,91]. Plants can be engineered to express proteins under the control of promoters that induce hairy root formation in plants. This rooting response results from the presence of the *rolABCD* genes from *Agrobacterium rhizogenes*, the bacterium that induces hairy root disease [92]. The rhizosecretion expression system harnesses the ability of *A. rhizogenes* to both target gene expression to the roots and to increase root biomass, subsequently increasing the amount of recombinant protein secreted into the soil [91]. Tobacco plants expressing a bacterial N₂O-cracking enzyme tagged for secretion under the control of the *A. rhizogenes rolD* promoter have been successful in demonstrating reducing activity [64,93]. Gas analysis was not performed to confirm that these plants mitigated N₂O emission. Ultimately, this approach arrives at a similar problem as other 'rhizoremediative' techniques: the N₂O-reducing ability of such a transgenic plant would be limited to the rhizosphere. This system would not have access to the bulk of N₂O gas, much of which comes from other sources;
 - (8) **Atmospheric phytoremediation using genetically engineered plants.** The potential of transgenic plants for environmental phytoremediation is well-documented: several fungal and bacterial oxidoreductases have been functionally expressed in plants as phytoremediation strategies including pentaerythritol tetranitrate reductase [94], mercuric reductase [95], and arsenate reductase [96]. This type of plant-based decontamination strategy provides advantages,

such as stable cultivation and control of the remediant organism and atmospheric exposure of the gas-cracking enzyme [97].

Atmospheric phytoremediation may ameliorate problems created by the other N₂O mitigation strategies described. The concept here is to develop crops with the ability to “crack” N₂O in both the soil and the atmosphere by incorporating the bacterial *nosZ* gene into their genomes. This gene encodes the nitrous oxide reductase enzyme (N₂OR), an oxidoreductase that catalyzes the removal of N₂O from the atmosphere, a process performed naturally by both denitrifying and non-denitrifying bacteria in the soil [98]. While conventional N₂O mitigation strategies aim to control N₂O production at earlier stages in the nitrification/denitrification pathway, this approach will target the atmospheric sum of N₂O emitted by all sources (Figure 2).

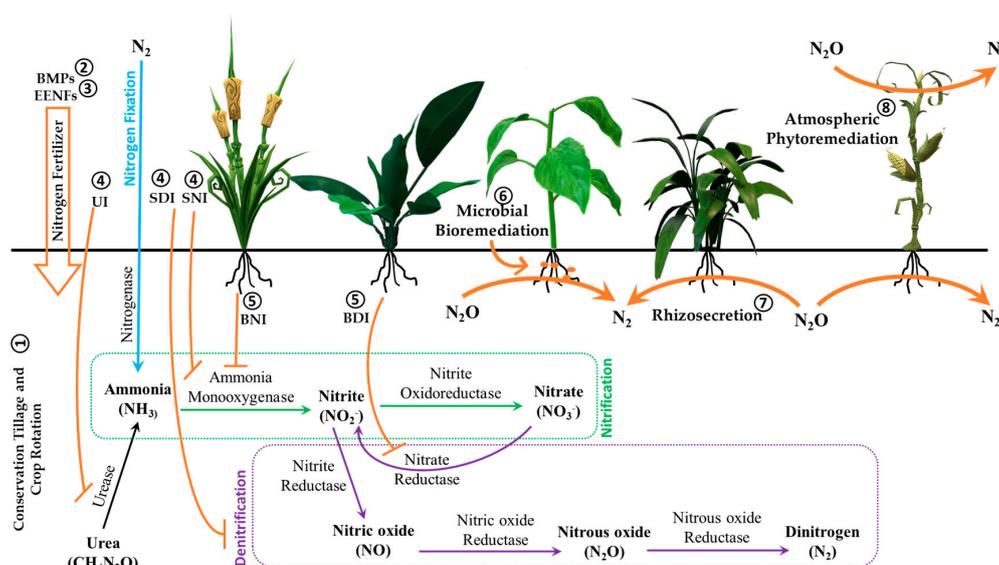


Figure 2. Nitrification-denitrification pathway and overview of current N₂O mitigation strategies. Orange arrows and lines show eight N₂O mitigation strategies described in Table 1. Green arrows show nitrification and purple arrows represent denitrification reactions. BDI, biological denitrification inhibitor; BMPs, best management practices; BNI, biological nitrification inhibitor; EENFs, enhanced efficiency nitrogen fertilizers; SDI, synthetic denitrification inhibitor; SNI, synthetic nitrification inhibitor; UI, urease inhibitor. **O** Encircled numbers refer to Table 1 strategies.

3. Nitrous Oxide Reductase—An Orphaned Soil Protein?

The *nosZ* gene can be categorized as either ‘clade I’ or ‘clade II’ based on sequence and *nos* operon organization, including the lack of an accessory *nosR* gene in the clade II members [99]. Clade II *nosZ* genes are also known as ‘atypical’ *nos* genes since they are found in non-denitrifying bacterial species. The N₂OR enzyme that the clade II gene encodes catalyzes the same reaction performed by the clade I-encoded enzyme, but has a higher affinity for N₂O [100], an important factor to consider when conceptualizing the development of an *nosZ*-expressing plant.

N₂OR is a multi-copper protein encoded by the *nosZ* gene (which is accompanied by an operon cluster of additional genes (*nosRDFYL*) [101]) and is the only enzyme that can catalyze the conversion of N₂O into N₂. The first active N₂OR was characterized from the soil bacterium *Pseudomonas stutzeri* and similar enzyme structures were resolved in bacterial species *Marinobacter hydrocarbonoclasticus* (formerly *Pseudomonas nautica*) (Figure 3), *Achromobacter cyclocastes*, and *Paracoccus denitrificans*. N₂OR is a head-to-tail homodimer and each monomer contains two domains: an electron transferring domain (binuclear Cu_A centre) and a catalytic domain (tetranuclear Cu_Z centre) [102]. There is some variability between the species regarding Cu_Z bridging and cupric coordination in the catalytic centre, suggesting that N₂OR substrate binding is species-specific. Regardless, the catalytic mechanism of N₂O reduction in N₂OR is still unclear [103].

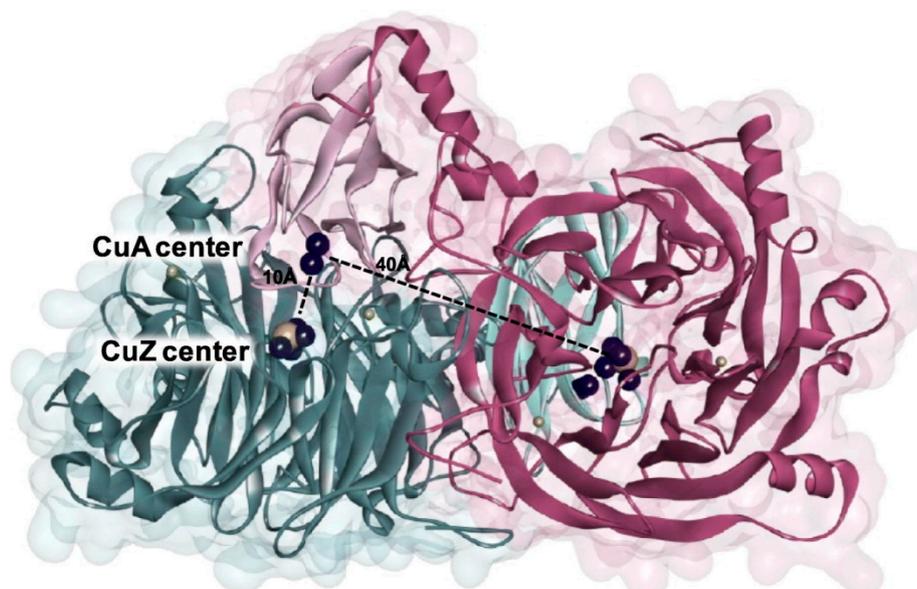


Figure 3. Structure of *Marinobacter hydrocarbonoclasticus* nitrous oxide reductase (N₂OR) homodimer. N₂OR is organized as a head-to-tail homodimer. Monomers are coloured differently so that they can be distinguished. In both monomers, the N-terminal domain is dark-coloured. The N-terminal domain forms a seven-bladed β -propeller fold that coordinates the catalytic tetranuclear active site Cu_Z through seven histidine residues at its hub. The C-terminal domain forms a cupredoxin fold and binds the dinuclear mixed-valent Cu_A centre [104].

The proven ability of N₂OR to “crack” the N₂O molecule raises the question of why the protein has not yet been incorporated into a commercially available transgenic cropping choice for environmentally motivated producers and small-plot farmers. Work has been done on this gene and its potential role in plant biotechnology since it was originally isolated in 1998 from the anaerobic soil bacterium *A. cyclocastes* [105,106], but it has yet to be converted into a commercially valuable tool. In this sense, N₂OR may be considered an “orphaned” protein, neglected among a veritable molecular toolkit of genes in the soil microflora [107,108]. Such forays into integrating soil and air sciences are demonstrative of the possibilities of what the soil microbiome offers biotechnologists [27]; it has already been discussed regarding the N-management possibilities offered by the microbiome and the current practice of ‘bioprospecting’ is also revealing a plethora of beneficial bacterial products, which is only accelerating thanks to whole-system approaches involving computational analyses [109].

Web of Science reports that between 1900 and 1991, there are no records binned under the combined topics “nitrous oxide reductase” and “microb*”. The scientific literature blossomed from its first occurrence of 1992 to the present day, witnessing at least 175 publications dealing with the science of this important enzyme in our total environment. The scientific community waited until 1996 to start discussing denitrification in a plant context, according to these same search terms. With the search terms “nitrous oxide reductase” and “plant”, the scientific record shows that soil microbiologists have taken a growing interest in the movement of N into the atmosphere (Figure 4). It is encouraging to note that in the same time period, the linkage between N₂OR and climate began its nascent phase.

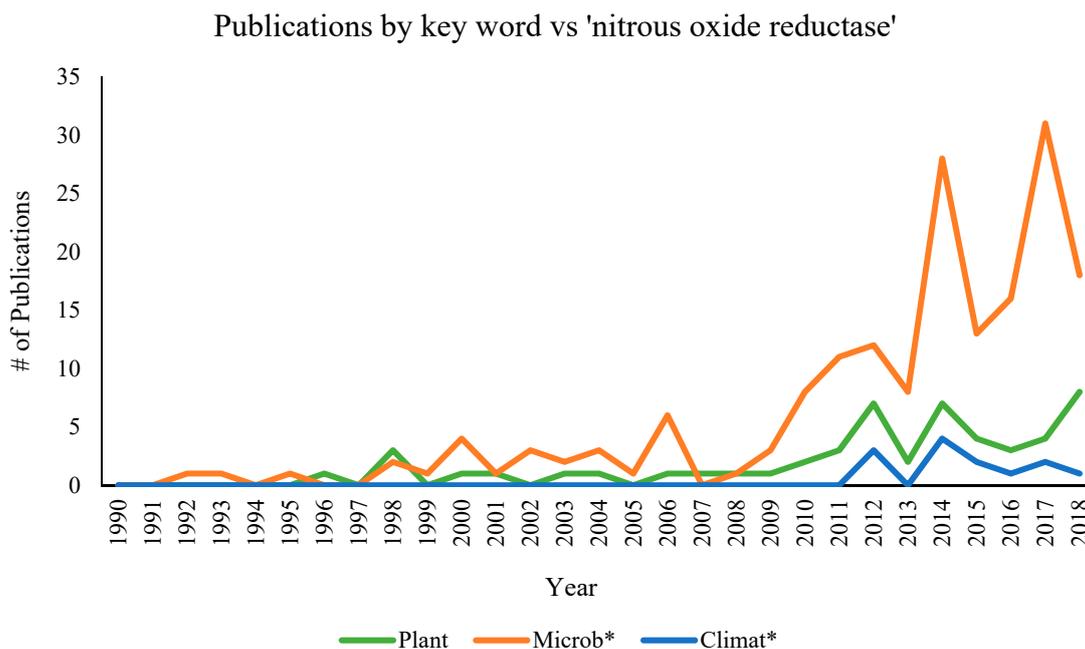


Figure 4. Nitrous oxide reductase-related publications released since 1990 on Web of Science (Clarivate Analytics). Publications by key word vs “nitrous oxide reductase” from 1990 to 2018. The orange line indicates “nitrous oxide reductase” + “microb*”; green: “nitrous oxide reductase” + “plant”; blue: “nitrous oxide reductase” + “climat*”.

4. Catch Me If You Can: Can Plants Catalytically Convert N_2O *in planta*?

Rather than a ‘cat and robin redbreast’ conundrum, we are confronted with an opportunity to deploy protein engineering to ensure that more N_2OR molecules are attracted to the substrate binding site of the copper enzyme. Protein engineering offers ways to sidestep the challenges of expressing a complex bacterial protein in a plant [110]. There are potential issues with a recombinant metalloprotein like N_2OR , such as whether the ABC transporter can assemble within a plant cell, or the plant can incorporate copper into the electron transferring and catalytic domains [111,112]. It is possible to re-engineer N_2OR and produce a functional product [66], so there is precedent for designing an artificial metalloenzyme through rational protein design. This approach may be key to engineering a plant-compatible N_2OR protein.

A principle challenge associated with imparting N_2OR functionality to plants is that transforming the *nosZ* sequence alone may not be effective [113]; in *P. stutzeri*, the transcription of *nosZ* was dependent on the *nosDFY* genes being expressed, as they encode components of a putative ABC transporter system for the biogenesis of the Cu_Z centre [114]. Therefore, catalytically active N_2OR may not be produced when only *nosZ* is expressed in a heterologous host [28]. Nevertheless, a model N_2O -expressing plant has been engineered [64,93]. The clade I *nosZ* gene from soil bacterium *Pseudomonas stutzeri* was successfully expressed in a heterologous system—in this case, the tobacco plant (*Nicotiana tabacum*). In those proof-of-concept experiments the *nosZ*-expressing tobacco plants reduced 826 $\mu g N_2O/min/gram$ of leaf tissue [115]. Assuming the tobacco yield to be 0.50 tonne/ha [116], the calculated N_2O -cracking ability of the *nosZ*-expressing tobacco could be as high as 600 kg of $N_2O/ha/day$ [115], or 60 tonnes/ha/year (100 day growing season). This value surpasses the calculated N_2O flux of 0.05–1.98 kg $N_2O/ha/year$ [117]. In other words, if every tobacco plant in the world produced N_2OR , this industrial crop (6.6 million tonnes were produced worldwide in 2016 [118]) could conceivably crack 785 Tg of N_2O (1 Tg = 1 million metric tonnes) during an average growing season of 100 days, far surpassing the estimated ~30 Tg of N_2O emitted per year [119]. Such catalytic capacity would give the ‘Stop Smoking’ campaigns a whole new flavour.

Although these transgenic plants produced a functional N₂OR enzyme, no gas analysis was performed to quantifiably ensure that these plants could reduce N₂O to N₂ using a recombinant N₂OR. In the future, it is imperative that such analyses be performed to properly judge the efficacy of such a gene-stacking trait system for atmospheric phytoremediation.

An associated issue rests with *P. stutzeri* being an anaerobic species that produces enzymes that function optimally in a low-oxygen environment. While expressing *nosZ* in plants to reduce N₂O appears to be an elegant solution, the N₂OR enzyme was not evolutionarily engineered to be functional in the presence of oxygen. Most soil bacteria that produce N₂OR do so in an anaerobic environment [102].

In the past five years, studies have identified several prokaryotic species that may express an oxygen-compatible N₂OR. Aerobic N₂O reducers may be undertaking an important role in mitigating the amounts of N₂O emitted to the atmosphere in events of oxic-to-anoxic transitions, but these systems have not yet been validated in plants. Here, we discuss two candidates for an oxygen-compatible *nosZ* expression system: clade II-*nosZ* member *Gemmatimonas aurantiaca* *gen nov.*, *spp. nov.* strain T-27, a polyphosphate-accumulating soil aerobe that is strongly represented in many oxygen-rich soil samples [120]; and *Azospira oryzae*, another clade II N-fixing bacterium originally isolated from the roots of rice (*Oryza sativa*) [121]. N₂O reduction by the *G. aurantiaca* strain T-27 was observed in both the absence and presence of oxygen [120]. The inability of this organism to consume N₂O in the complete absence of oxygen and the observed oxygen-induced activation of *nosZ* expression compels one to consider *in planta* overexpression, whereby the diurnal fluctuation of photosynthetic oxygen production may offer an egress for N₂O accumulation. The *A. oryzae* strains I09 and I13 also show more rapid N₂OR recovery rates and tolerance against oxygen inhibition than *P. stutzeri* [121] and so may be appropriate candidates for crop plant transformation and N₂OR expression.

If the ideal *nosZ* sequence were to be identified and transformed into commercially important crop plants, the benefits would be numerous and profound: seed-borne GHG technology foresees the transgenic cassette passed on from generation to generation, meaning that constant application of the beneficial catalyst would not be required (as with NI application and rhizoremediation); the expression of *nosZ* in the aerial tissues of the plants allows the reducing enzyme to confront N₂O much more easily than when the enzyme is expressed in the soil.

5. Novel Breeding Task: “Gas Cracking” Plants

The challenge of expressing heterologous bacterial proteins in plants necessitates codon optimization due to differences in GC content and codon bias with eukaryotes [122]. Altering the codon bias (or applying ‘directed evolution’ [123]) of a bacterial gene to be expressed in plants has been highly successful: *P. stutzeri nosZ* in tobacco [115], 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase from *Agrobacterium tumefaciens* in Roundup Ready crops [124], and *Bacillus thuringiensis Cry* genes in maize [125] and rice [126]. Indeed, the global advance promulgating engineered crops is pillared on today’s artificial intelligence-guided plant codon optimization rules offered by both large and small boutique DNA houses. However, there has been success expressing native bacterial sequences in plants, i.e., in the case of cotton expressing the native sequence of the *P. stutzeri* gene *ptxd* (PHOSPHONATE DEHYDROGENASE) [127,128]. One can dare to fathom how a universally-functional *nosZ* expression system could conceivably redirect some aspects of GHG mitigation research. Such a plant transformation cassette could theoretically be applied to any plant—wheat, rice, soybean, peat moss [129]—recruiting these species for the purpose of denitrification mitigation.

Even with an effective *nosZ* expression system, there are additional challenges in developing *nosZ*-expressing plant lines. There are relatively few powerful monocot-optimized expression systems available [130] (although *Bt* corn, LibertyLink wheat, and Roundup Ready wheat can attest to the effectiveness of the 35S promoter system in monocots), and there is difficulty in transforming monocots [65]. With the advent of new plant transformation technologies like the soil bacterium

Ochrobactrum haywardense [131] and the *BABYBOOM/WUSCHEL2* system [132], the production of genetically modified crops with stacked or pyramided GHG genes may be expedited in the near future.

6. Conclusions—Challenges to the Future Success of *nosZ*

We must address what may be the greatest challenge of all for the modern molecular plant breeder: convincing the general public that transgenic crops may be beneficial for all the plant-planet's denizens, as modified crops that enter the food stream may appear unpopular in some boroughs. Regardless, there is a clear, urgent need to control soil N₂O losses due to the detrimental effects of this potent GHG in the atmosphere. Climate-smart crops should be given a crack at directly addressing this issue and tackling climate change. Such GHG-reducing plant lines, endowed with the ability to catalytically “crack” N₂O in the air, could be vital in the battle to shift public perception towards the acceptance of “GMOs” in agricultural research.

Involvement of N₂O in climate change and global warming has been the subject of increasing investigations due to its potential heat-trapping properties [3]. N₂O emission from soil is primarily the result of an incomplete enzymatic reaction which is mediated by the bacterial enzyme, N₂OR [98]. Therefore, in the late 1990s [105,106], the development of N₂OR-positive transgenic plants was proposed as an environmental phytoremediation strategy with promise to remove N₂O from soil and the atmosphere (Figure 2). However, producing a foreign protein in a plant cell is often a serious challenge. For example, different codon usage [133] and cellular properties between eukaryotic and prokaryotic cells are considered as unknown aspects of this strategy. At least two key questions need to be addressed in future studies to probe the probability for success of this green gene de-toxic tactic for accelerating the destruction of nitrous oxide via canopy catalysis: (1) Which candidate is the best source-organism to donate *nosZ* sequence for plant transformation? Activity of bacterial N₂OR is associated with the anaerobic conditions in soil [101], whereas the plant cell is mostly an aerobic environment. Photosynthesis and respiration cause different levels of oxygen content in plant cells in a diurnal cycle which is not consistent with the enzymatic activity of N₂OR in anaerobic soil bacteria. Therefore, selecting obligate or facultative aerobic bacteria containing active N₂OR enzymes as ‘the source code’ would be pivotal; (2) Which plant cell compartment is the best destination for targeting N₂OR accumulation? The native enzyme N₂OR in bacteria is directed to the periplasm, where Cu chaperones provide enough Cu for the assembly of metal centres [134]. The absence of periplasmic space in plant cells reinforces the notion that subcellular localization of N₂OR may influence its enzymatic activity *in planta*. Moreover, the important role of Cu in the functional assembly of N₂OR posits whether the transformation of bacterial *nosDFY*, along with *nosZ*, is essential for a functional enzyme. Urgent exploration of how the cellular pool of metal nutrients and proteins (pseudo chaperones) in eukaryotic cells may suffice to activate N₂OR *in planta* may compel the use of such climate-smart plants.

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