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# Potential Pathway of Nitrous Oxide Formation in Plants

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Plants can produce and emit nitrous oxide ($N_2O$), a potent greenhouse gas, into the atmosphere, and several field-based studies have concluded that this gas is emitted at substantial amounts. However, the exact mechanisms of $N_2O$ production in plant cells are unknown. Several studies have hypothesised that plants might act as a medium to transport $N_2O$ produced by soil-inhabiting microorganisms. Contrarily, aseptically grown plants and axenic algal cells supplied with nitrate ($NO_3$) are reported to emit $N_2O$, indicating that it is produced inside plant cells by some unknown physiological phenomena. In this study, the possible sites, mechanisms, and enzymes involved in $N_2O$ production in plant cells are discussed. Based on the experimental evidence from various studies, we determined that $N_2O$ can be produced from nitric oxide (NO) in the mitochondria of plants. NO, a signaling molecule, is produced through oxidative and reductive pathways in eukaryotic cells. During hypoxia and anoxia, $NO_3$ in the cytosol is metabolised to produce nitrite ($NO_2$), which is reduced to form NO *via* the reductive pathway in the mitochondria. Under low oxygen condition, NO formed in the mitochondria is further reduced to $N_2O$ by the reduced form of cytochrome c oxidase (CcO). This pathway is active only when cells experience hypoxia or anoxia, and it may be involved in $N_2O$ formation in plants and soil-dwelling animals, as reported previously by several studies. NO can be toxic at a high concentration. Therefore, the reduction of NO to $N_2O$ in the mitochondria might protect the integrity of the mitochondria, and thus, protect the cell from the toxicity of NO accumulation under hypoxia and anoxia. As $NO_3$ is a major source of nitrogen for plants and all plants may experience hypoxic and anoxic conditions owing to soil environmental factors, a significant global biogenic source of $N_2O$ may be its formation in plants *via* the proposed pathway.

**Keywords: anoxia, hypoxia, nitrate, nitrite, nitric oxide, nitrous oxide, mitochondrion**

## INTRODUCTION

Nitrous oxide ($N_2O$) is a potent greenhouse gas, and its potential to increase global warming is approximately 300-fold higher than $CO_2$ (Tian et al., 2018). Globally, the primary sources that release $N_2O$ into the atmosphere are soil, ocean, manure application, industries, and biomass burning (Thomson et al., 2012). The nitrification and denitrification processes, mainly mediated by certain groups of soil micro-organisms (Hu et al., 2015), account for more than two-thirds of its emission into the atmosphere (Thomson et al., 2012). These processes are considerably increased by human activities, leading to an increase in the production of $N_2O$ in the soil, and thus, its concentration in the atmosphere. Increased level of $N_2O$ in the atmosphere has significantly contributed to global warming (Tian et al., 2018); therefore, understanding the pathways of $N_2O$ formation in various sources is essential for mitigating these effects.

Key pathways involved in $N_2O$ production in microbes include nitrification, nitrifier denitrification, nitrification-coupled denitrification, and denitrification (Baggs, 2011; Hu et al., 2015; Tian et al., 2018). However, there seems to be a gap between source estimation and the global $N_2O$ budget, leading to a high level of uncertainty in the budget estimations (Davidson and Kanter, 2014). This gap may be because not all sources of $N_2O$ to the atmosphere are accounted for (Syakila and Kroeze, 2011). Therefore, it is necessary to understand all sources of $N_2O$ and underlying mechanisms to elucidate its global budget. The production of $N_2O$ in axenic microalgae (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Plouviez et al., 2017) and ascetically grown plants (Goshima et al., 1999; Hakata et al., 2003) indicates that it could be produced by higher organisms and that the processes might be different from those in micro-organisms. Algae and plants are not included as sources of $N_2O$ (Syakila and Kroeze, 2011; Lenhart et al., 2019; Plouviez et al., 2019), but they might be the missing sources of $N_2O$, causing high uncertainties in the global budget.

The roles of plants in $N_2O$ emission to the atmosphere are diverse. Plants can not only modify soil characteristics and subsequently influence $N_2O$ production in the soil (Gao et al., 2019) but also produce it in significant amounts and release it to the atmosphere (Lenhart et al., 2019). Thus, understanding the pathway of $N_2O$ formation and the contribution of plants to total emission is essential to accurately estimate the global $N_2O$ budget. Several field-based studies have hypothesised that $N_2O$ emitted by plants is produced in soil by microorganisms (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017). In this theory, plants are considered just a medium to transport $N_2O$ produced by soil microorganisms; however, laboratory-based studies have provided clear evidence that plants produce and emit $N_2O$ although the underlying mechanisms are unknown (Goshima et al., 1999; Hakata et al., 2003; Lenhart et al., 2019). Therefore, $N_2O$ emitted by plants might originate from two sources, namely, the soil microorganisms and plants.

Studies, which have hypothesised that plant-emitted $N_2O$ is produced by soil microorganisms, have only measured the fluxes from plants and concluded that plant-emitted $N_2O$ might be produced by soil microorganisms. $N_2O$ produced in plant cells might also use the same pathway, that is, transpiration, to release it to the atmosphere. This raises the question whether measuring the fluxes alone provides substantial evidence to prove the hypothesis, because flux measurement methods can just estimate the emission of $N_2O$ and cannot distinguish the sources. More robust methods such as isotope studies would provide more insights to distinguish the sources of $N_2O$. For examples, injecting $^{15}N$-$N_2O$ into the root zone and measuring the subsequent fluxes would elucidate whether plants are a medium for $N_2O$ transport or not. However, no study has injected $^{15}N$-labeled $N_2O$ into the soil zone and measured the subsequent $N_2O$ emission from plants. Moreover, more powerful tools such as site preference (SP) measurement would provide insights to distinguish the sources of $N_2O$ emitted by plants under field conditions.

In the natural environment, if plant emitted N2O constitute significant amount of both sources (soil micro-organisms and plant cells produced N2O), it will be highly challenging to distinguish the portion of the sources. A recent field experiment reported considerably lower $N_2O$ concentrations in soil water than in tree stems (Ward et al., 2019). Similarly, plants exposed to $NH_4$ did not emit $N_2O$ despite the high rate of $N_2O$ production in the rhizosphere (Smart and Bloom, 2001), indicating that $N_2O$ emitted by plants might not be produced by soil microorganisms and that $N_2O$ emitted through transpiration might be a less significant process than $N_2O$ production in plants. Furthermore, the hypothesis that plants are just a conduit for soil microorganisms-produced $N_2O$ is not supported by a recent study of Lenhart et al. (2019). They provided new evidence that dual isotopocule fingerprints of $N_2O$ emitted by plants differed from that produced by all known microbial or chemical processes, indicating that plant-emitted $N_2O$ is produced in plant cells.

Although plants are known to produce $N_2O$ and emit it to the atmosphere, the exact mechanisms of $N_2O$ production in plant cells are unknown (Goshima et al., 1999; Hakata et al., 2003; Lenhart et al., 2019). This might be the reason that most studies on $N_2O$ fluxes in plants (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017) have hypothesised that plant parts act as a conduit for soil-produced $N_2O$. Studies, which have claimed that plants could produce $N_2O$, have not elucidated a possible production pathway. Therefore, the main objective of this study was to review the possible pathway of $N_2O$ formation in plant cells.

## PATHWAY OF $N_2O$ FORMATION IN PLANT CELLS

### Nitrate ($NO_3$) as a Precursor for $N_2O$ Formation in Plant Cells

Nitrogen (N) is an essential macronutrient influencing cell metabolism (O'Brien et al., 2016). Plants can use several forms

of N from the soil; however, $NO_3$ and ammonium ($NH_4$) are the major forms of inorganic N that are readily available for plant uptake (O'Brien et al., 2016; Hachiya and Sakakibara, 2017). $NO_3$ is a major source of N for plants in agricultural and natural soils (von Wirén et al., 2000), due to its high soil concentration and diffusion coefficients, making it readily available to plant roots (Miller and Cramer, 2005). After absorption, $NO_3$ is directly reduced in the root, transported to the leaf for reduction (Maathuis, 2009; Hachiya and Sakakibara, 2017), or stored in the vacuoles and remobilised when the external supply is limited (van der Leij, et al., 1998; Fan et al., 2007), making it an essential macronutrient in plant metabolism.

$NO_3$ is a major source for $N_2O$ formation in both soil (Thomson et al., 2012) and plants (Goshima et al., 1999; Smart and Bloom, 2001; Hakata et al., 2003; Lenhart et al., 2015; Lenhart et al., 2019). Isotope labeling methods have demonstrated that plants as well as other eukaryotic organisms emit $N_2O$, only when supplied with $NO_3$. For example, when $^{15}N$-labeled $NO_3$ was supplied as a source of N to various species of plants (Goshima et al., 1999; Smart and Bloom, 2001; Lenhart et al., 2019), lichens (Lenhart et al., 2015), and animals (Stief et al., 2009), $^{15}N$-labeled $N_2O$ was emitted, but when the N source was $^{15}N$-labeled $NH_4$, there was no $N_2O$ emission. This evidence clearly shows that $NO_3$ is the precursor of $N_2O$ in lichens, higher plants, and animals. Therefore, if plants were just a medium of transportation of soil-produced $N_2O$ as hypothesised by many studies (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017), aseptically grown plants would not have emitted $N_2O$ when supplied with $NO_3$ (Goshima et al., 1999; Hakata et al., 2003). Similarly, if $N_2O$ emitted by plants is produced by microorganisms (nitrifying and denitrifying bacteria), $NH_4$ supplementation should contribute to $N_2O$ emission from plants and aseptically grown plants should not emit $N_2O$. As $N_2O$ was not emitted when plants were supplied with $NH_4$ and aseptically grown plants emitted $N_2O$ (Goshima et al., 1999; Hakata et al., 2003), we predicted that $NO_3$ metabolism in a cell might play a role in $N_2O$ formation in plants. Moreover, the processes in the soil microbial communities and higher organisms may be different, or the denitrification process may be common between microorganism and plants, as $NO_3$ is the substrate for denitrification.

## Nitrite ($NO_2$) Derived From $NO_3$ Reduction Is the Precursor of $N_2O$ in Plant Cells

After the uptake of $NO_3$ by plant roots, it is reduced to $NO_2$ in plant cells by a cytosolic enzyme called nitrate reductase (NR) (Chamizo-Ampudia et al., 2017). However, in animals, $NO_3$ from food is reduced by bacteria in the digestive tracks (Lundberg et al., 2008). Moreover, germ-free mice are reported to possess NR activity, and the activity is catalysed by xanthine oxidoreductase, which is significantly high in the gastrointestinal tissues, compared with other tissues (Jansson et al., 2008). Under normal conditions, $NO_3$ absorbed by the roots is reduced to $NO_2$ by the NR, and then nitrite reductase (NiR) catalyses the reduction of $NO_2$ to $NH_4$, which is incorporated into amino acids (Oliveira and Sodek, 2013; Plouviez et al., 2017). However, under hypoxic and anoxic conditions, root $NO_3$ uptake increases with the activation level of NR (Botrel and Kaiser, 1997; Rockel et al., 2002; Morard et al., 2004; Horchani et al., 2010). Furthermore, $NO_2$ accumulates in the cytoplasm of cells (Allègre et al., 2004; Morard et al., 2004), as both hypoxia and anoxia suppress the reduction of $NO_2$ to $NH_4$ (Botrel and Kaiser, 1997). A $^{15}N$ isotope labeling study has showed that $^{15}N$-$NO_2$ assimilation into amino acids is sharply reduced under hypoxic conditions (Oliveira and Sodek, 2013). The accumulated $NO_2$ in the cytoplasm enters the mitochondria with the help of proteins in the chloroplast (Sugiura et al., 2007; Gupta and Igamberdiev, 2011). Moreover, mitochondrial inner membrane anion channels may import $NO_2$ to the mitochondria (Gupta and Igamberdiev, 2011).

Not only $NO_3$, but also $NO_2$ is widely reported to be a precursor of $N_2O$ in eukaryotic organisms. Using $^{15}N$ isotope labeling method, it has been demonstrated that $NO_2$ is another precursor of $N_2O$ formation in plants and algal cells. For example, when $^{15}N$-labeled $NO_2$ was supplied to aseptically grown tobacco plants (Goshima et al., 1999; Hakata et al., 2003) and algal systems (Weathers, 1984), they emitted $^{15}N$-$N_2O$. Furthermore, axenic algae supplied with $NO_2$ produced $N_2O$ (Guieysse et al., 2013; Plouviez et al., 2017). The enzyme NR has been proved to play a role in $N_2O$ production in plants. For example, when tobacco plants were supplied with $NO_3$ and tungstate (NR inhibitor), $N_2O$ production was inhibited in the plants (Goshima et al., 1999). As $NO_2$ also contributes to $N_2O$ production in plants (Goshima et al., 1999; Hakata et al., 2003), NR might indirectly be involved in $N_2O$ formation by catalysing the reduction of $NO_3$ to $NO_2$. A similar role of NR has been observed in algae (Plouviez et al., 2017). However, NiR-deficient transgenic plants and algae have been reported to produce $N_2O$ when supplied with $NO_2$ (Hakata et al., 2003; Plouviez et al., 2017), and this suggests that the pathway of $NO_2$ reduction to $NH_4$ is not involved in $N_2O$ production in plants and algal cells. Additionally, $NO_3$, NR, and $NO_2$ are involved in $N_2O$ production in plants and algal cells, but NiR and $NH_4$ are not involved in the $N_2O$ production pathway. This indicates that $NO_2$ has to be transported to other cell organelles rather than plastid. Overall, the available evidence indicates that exogenous $NO_2$ along with endogenous $NO_2$ derived through $NO_3$ reduction in the cytosol by NR plays a role in $N_2O$ formation in plant cells.

## Mitochondrial Reduction of Nitrite to NO

We previously discussed $NO_3$ reduction to $NO_2$ in the cytosol. NO has several essential roles in plant and animal cells (Wendehenne et al., 2001), and the conversion mechanisms of $NO_2$ to NO are well established in eukaryotic cells. In plants, NO can be produced in the chloroplast, peroxisomes, and mitochondria by either oxidative or reductive pathways (Rőszer, 2012). The oxidative pathway is dependent on L-arginine, polyamine, or hydroxylamine, whereas the reductive pathway is dependent on $NO_3$ and $NO_2$ (Benamar et al., 2008; Lundberg et al., 2008; Gupta et al., 2011; Gupta and Igamberdiev,

2011; Astier et al., 2018). The oxidative pathway of NO formation is dominant when the oxygen supply to cells is sufficient, whereas the reductive path is dominant under hypoxic conditions. By shifting from the oxidative to reductive pathway, the cells maintain the level of NO along with the physiological and pathological oxygen and proton gradients (Lundberg et al., 2008). It may be essential to shift processes, as plants may experience hypoxia due to soil environmental conditions.

In plant cells, $NO_2$ assimilation to $NH_4$ by the NiR enzyme is a well-known pathway of $NO_2$ metabolism. As $NO_2$ addition can lead to $N_2O$ formation in plants (Goshima et al., 1999; Hakata et al., 2003) and NiR-deficient plants can produce $N_2O$ (Hakata et al., 2003), we suggest that $NO_2$ is metabolised by another pathway in plants to produce $N_2O$. Although the mechanisms of $NO_2$ transport to the mitochondria are not precise, it is evident that the mitochondria are a site of reduction of $NO_2$ to NO. For example, the mitochondria have been reported to reduce $NO_2$ to NO under hypoxic and anoxic conditions in fungi (Kobayashi et al., 1996; Castello et al., 2006), algae (Tischner et al., 2004; Calatrava et al., 2017), plants (Gupta et al., 2005; Planchet et al., 2005; Benamar et al., 2008; Gupta and Kaiser, 2010), and animals (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Kozlov et al., 1999; Castello et al., 2006; Ascenzi et al., 2014). However, the enzymes involved in the mitochondrial reduction of $NO_2$ to NO are not clear. For example, mitochondria that lack NiR can reduce $NO_2$ to NO in animals and plants (Gupta and Igamberdiev, 2011). Nitric oxide synthases (NOS) have been reported to be present in plant (Guo and Crawford, 2005) and animal mitochondria (Giulivi et al., 1998). However, the NOS activity in the mitochondria of plants is questioned (Moreau et al., 2008; Gupta and Kaiser, 2010). Tischner et al. (2004) identified an alternative oxidase (AOX) in the mitochondria as a catalyser of the reduction of $NO_2$ to NO under anoxic conditions. The mitochondrial respiratory chain is responsible for NO production using $NO_2$ as the substrate under low pH, hypoxic, or anoxic conditions (Castello et al., 2006). Mitochondrial and bacterial electron transport chains (ETCs) are involved in NO production from $NO_2$ under hypoxic conditions than under normoxic conditions (Horchani et al., 2011). Under hypoxic conditions, $NO_2$ is reduced to NO at complex III in the mitochondria of pea plants (Benamar et al., 2008). Ascenzi et al. (2014) reported cytochrome c in horse heart cells and bovine heart reduced $NO_2$ to NO, and the activity was high under anoxic and acidic conditions (Basu et al., 2008). The mitochondrial molybdopterin enzymes in the reduced form catalyse the reduction of $NO_2$ to NO, and the rate was increased when the pH was decreased from 7.5 to 6.5 (Jakobs et al., 2014; Sparacino-Watkins et al., 2014; Maia and Moura, 2015; Bender and Schwarz, 2018). Furthermore, cytochrome reductase in tobacco plants can reduce $NO_2$ to NO (Alber et al., 2017). Although at the molecular level, the reductive pathway for NO formation is well documented, at the field scale, the emission of NO is less documented. For instance, when plants were supplied with $NO_3$, NO was emitted under anoxic conditions (Klepper, 1987; Rockel et al., 2002). The leaf $NO_2$ level and NO emission under anoxic conditions were significantly higher than those under normoxic conditions (Rockel et al., 2002). These findings suggest that $NO_2$ can be reduced to NO in the mitochondria; however, the involvement of various enzymes within the mitochondria raises the question whether these enzymes catalyse the reduction process simultaneously or they function differently under varied cell environment.

## NO Conversion to $N_2O$ in the Mitochondria

NO is a signaling molecule in cells, and several studies have focused on its formation in the mitochondria. However, studies on the reduction of NO to $N_2O$ in the mitochondria are limited, although there is a strong indication that this process exists (Gupta and Igamberdiev, 2011). The inner membrane of the mitochondria has an enzyme called cytochrome c oxidase (CcO). The primary function of CcO is to reduce $O_2$ to $H_2O$ (Collman et al., 2007; Blomberg and Ädelroth, 2018). Moreover, CcO has several other functions, such as the oxidisation of NO formed in the mitochondria to $NO_2$ (Brudvig et al., 1980; Zhao et al., 1995; Pearce et al., 2002; Taylor and Moncada, 2010). Furthermore, the reduced form of CcO can catalyse the reduction of NO to $N_2O$ (Brudvig et al., 1980; Zhao et al., 1995). Thus, either oxidation or reduction of NO by CcO results in the metabolism of NO with safe end products. The similar properties of $O_2$ and NO facilitate the binding of NO to CcO, and this activity is pronounced under oxygen-limited conditions (Ghafourifar and Cadenas, 2005). The mitochondrial electron transport chain (ETC) in axenic algae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*) catalyses the reduction of NO to $N_2O$ (Guieysse et al., 2013; Plouviez et al., 2017; Plouviez et al., 2019). CcO has some rudimentary nitric oxide reductase activity, and therefore, when NO is the substrate instead of $O_2$, two molecules of NO yield $N_2O$ and $H_2O$ (Brudvig et al., 1980; Zhao et al., 1995; Koivisto et al., 1997; Igamberdiev et al., 2010; Blomberg and Ädelroth, 2018; Poderoso et al., 2019). It has also been proven isotopically that NO is reduced to $N_2O$ by CcO in higher organisms (Brudvig et al., 1980). As mitochondrial CcO has evolved from denitrifying enzymes, under hypoxic conditions in cells, the mitochondrial CcO can still reduce NO to $N_2O$ (Saraste, 1994; Saraste and Castresana, 1994). Furthermore, another enzyme in the mitochondria, that is, quinone of the ETC catalyses NO reduction to $N_2O$ (Alegria et al., 2004; Igamberdiev and Hill, 2009; Sanchez-Cruz and Alegría, 2009). Therefore, mitochondria can be a potent site of $N_2O$ formation under oxygen-limited conditions, and it should be a focus of future research.

Similar to the observations in plants, macrofauna and earthworms are also found to emit $N_2O$ when supplied with $NO_3$ and under $O_2$-limited conditions (Horn et al., 2003; Stief et al., 2009). Earthworms do not produce $N_2O$ when supplied $NH_4$ (Horn et al., 2003). Moreover, in other studies, listed in **Table 1**, when $^{15}N$-labeled $NH_4$ was used as a substrate, there was no $N_2O$ emission. This shows that $NO_3$ metabolism at the cellular level produces $N_2O$ in both plants and animals. As described above, the ETC in (**Figure 1**) mitochondria can reduce NO to $N_2O$ under less oxic conditions, which suggests

that $N_2O$ emitted by earthworms and macrofauna (Horn et al., 2003; Stief et al., 2009) might also be produced from hypoxic mitochondria. The gut of insects has a hypoxic environment (Johnson and Barbehenn, 2000), which may explain the higher level of $N_2O$ production in the gut (Stief et al., 2009). Moreover, axenic algae supplied with $NO_2$ produced significantly higher levels of $N_2O$ under dark conditions than under light conditions (Guieysse et al., 2013; Plouviez et al., 2017). The low emission of $N_2O$ under light conditions may be due to the supply of photosynthetic $O_2$ to the cells.

Based on experimental evidence gathered from various studies, we propose that the reductive pathway of NO formation in the mitochondria and further reduction of NO by the mitochondrial ETCs contributes to the formation of $N_2O$ (in eukaryotic cells, as presented in **Figure 1**). The process is catalysed by various enzymes, and it might be pronounced under hypoxic and anoxic conditions but not under normoxic conditions. The proposed pathway is further supported by the existence of a denitrifying pathway, and the associated enzymes and genes in *Globobulimina* species and the localisation of enzymes in the mitochondria (Woehle et al., 2018). As higher animals possess well developed respiratory and circulatory systems that transport $O_2$, they may not experience hypoxia. However, plants lack such sophisticated systems to transport $O_2$ (Voesenek et al., 2016), and therefore, may experience hypoxia and anoxia that favour $N_2O$ formation. Field studies have reported high $N_2O$ emission from plants under flooded conditions (Rusch and Rennenberg, 1998; Machacova et al., 2013), suggesting the role of hypoxia and anoxia in $N_2O$ formation in plants.

## SIGNIFICANCE OF $N_2O$ FORMATION *VIA* THE $NO_3$-$NO_2$-NO PATHWAY IN PLANTS

$NO_3$ is not only a major nutrient in plant cells but also a signaling molecule (Zhao et al., 2018). Several studies have reported that $NO_3$ plays a role in hypoxia tolerance. For example, $NO_3$ maintains the growth of plants under oxygen-limited conditions, and its absence disturbs plant growth (Horchani et al., 2010). Anoxia tolerance of tomato plant is enhanced by nitrate reduction (Allègre et al., 2004). Moreover, anoxia strongly induces NR activity and the induced NR

**TABLE 1 |** Compilation of the substrates, mediums and products that used labeled N sources and their subsequent measurements of $N_2O$ emissions.

| | Substrate | Medium | Product | Reference |
|---|---|---|---|---|
| 1. | $^{15}N$ labeled $NO_3$<br>$^{15}N$ labeled $NH_4$ | Aseptically grown tobacco plants | $^{15}N$ labeled $N_2O$<br>No $N_2O$ emission | Goshima et al. (1999) |
| 2. | $^{15}N$ labeled $NO_3$<br>$^{15}N$ labeled $NH_4$ | Lichen | $^{15}N$ labeled $N_2O$<br>No $N_2O$ emission | Lenhart et al. (2015) |
| 3. | $^{15}N$ labeled $NO_3$<br>$^{15}N$ labeled $NH_4$ | Wheat plant | $^{15}N$ labeled $N_2O$<br>No $N_2O$ emission | Smart and Bloom (2001) |
| 4. | $^{15}N$ labeled $NO_3$ | Soybean plant | $^{15}N$ labeled NO and $N_2O$ | Dean and Harper (1986) |
| 5. | $^{15}N$ labeled $NO_3$ | Macro fauna | $^{15}N$ labeled $N_2O$ | Stief et al. (2009) |
| 6. | $^{15}N$ labeled $NO_2$ | Tobacco plant | $^{15}N$ labeled $N_2O$ | Goshima et al. (1999) |
| 7. | $^{15}N$ labeled $NO_2$ | Aseptically grown tobacco plant | $^{15}N$ labeled $N_2O$ | Hakata et al. (2003) |
| 8. | $^{15}N$ labeled $NO_2$ | Axenic algae | $^{15}N$ labeled $N_2O$ | Weathers (1984) |
| 9. | $^{15}N$ labeled NO<br>$^{14}N$ labeled NO | Reduced form of beef heart cytochrome c oxidase (CcO) | $^{15}N$ labeled $N_2O$<br>$^{14}N$ labeled $N_2O$ | Brudvig et al. (1980) |

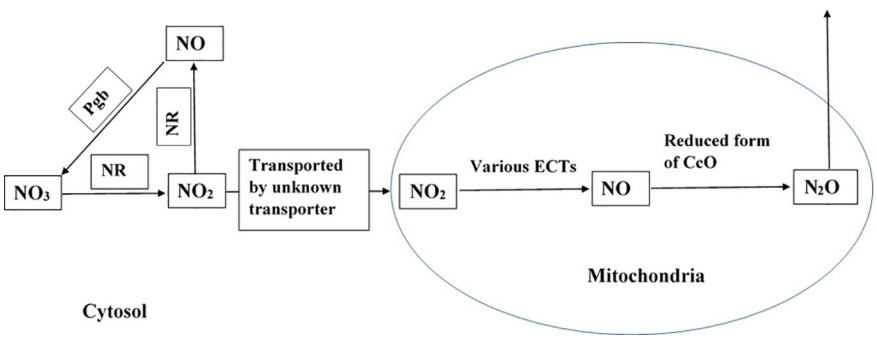


**FIGURE 1 |** Potential pathway of $N_2O$ formation in plant cells. NR represents nitrate reductase and Pgb represents phytoglobin (Brudvig et al., 1980; Zhao et al., 1995; Goshima et al., 1999; Gupta and Kaiser, 2010; Guieysse et al., 2013; Plouviez et al., 2017). The pathway is active in presence of $NO_3$ and $NO_2$, and when plants experience hypoxia and anoxia.

activity prevents pH from dropping to life-threatening levels (Allègre et al., 2004). $NO_3$ nutrition in plants decreases the total respiration rate and reactive oxygen species levels (Wany et al., 2019), but increases ATP production under hypoxic conditions (Stoimenova et al., 2007; Wany et al., 2019). Under oxygen-limited conditions, $NO_3$ protects the ultrastructure of mitochondria (Vartapetian et al., 2003). The addition of $NO_3$ to the root zone of plants released significantly less amount of ethanol compared with roots supplied with $NH_4$ under hypoxic conditions (Oliveira et al., 2013). This suggests $NO_3$ plays an important role to decrease alcoholic fermentative metabolism in plants during hypoxia (Oliveira et al., 2013). Overall, these findings suggest that $NO_3$ and NR play an important role to maintain the integrity of plant cells under oxygen-limited conditions.

$NO_2$ is also reported to play important roles under oxygen-limited conditions in plants. Benamar et al. (2008) found that $NO_2$-dependent NO production in the mitochondria can regulate surrounding $O_2$ level. Moreover, plant mitochondria can synthesise ATP under anaerobic conditions when supplied with $NO_2$ (Stoimenova et al., 2007; Gupta et al., 2016). The supply of $NO_2$ decreased lipid peroxidation and reactive oxygen species formation (Gupta et al., 2016). The absence of $NO_2$ as a terminal acceptor for ETC during hypoxia leads to mitochondrial depolarisation (Gupta et al., 2016). $NO_2$ supplemented roots released significantly less amount of fermentative ethanol during hypoxia than $NH_4$-supplemented roots (Oliveira et al., 2012; Oliveira et al., 2013), suggesting the vital role of $NO_2$ in plants to survive under oxygen-limited conditions.

NO helps plants to cope under several environmental stresses. For example, NO is essential for the homeostasis of $O_2$ level in plants under oxygen-limited conditions (Gupta and Igamberdiev, 2011). NO production in the mitochondria has several implications in plants as illustrated in **Figure 2**. For example, NO can break seed dormancy and stimulate seed germination in plants (Beligni and Lamattina, 2000; Bethke et al., 2004). Similarly, under hypoxic stress, NO is vital for the formation of aerenchyma in the roots (Wany et al., 2017). NO production in the mitochondria under low-oxygen conditions can help in ATP synthesis, preventing excessive depletion of energy (Stoimenova et al., 2007). $NO_3$-$NO_2$-dependent NO production in plant roots decreases fermentative ethanol production during hypoxia (Oliveira et al., 2012; Oliveira et al., 2013).

Although NO has been well established as a signaling molecule, its high concentration in cells leads to cell death (Boscá and Hortelano, 1999; Brown and Borutaite, 2002). Therefore, it is critical to regulate its concentration in cells, as a higher amount of NO is formed under hypoxic and anoxic conditions. Two mechanisms are reported to occur in the

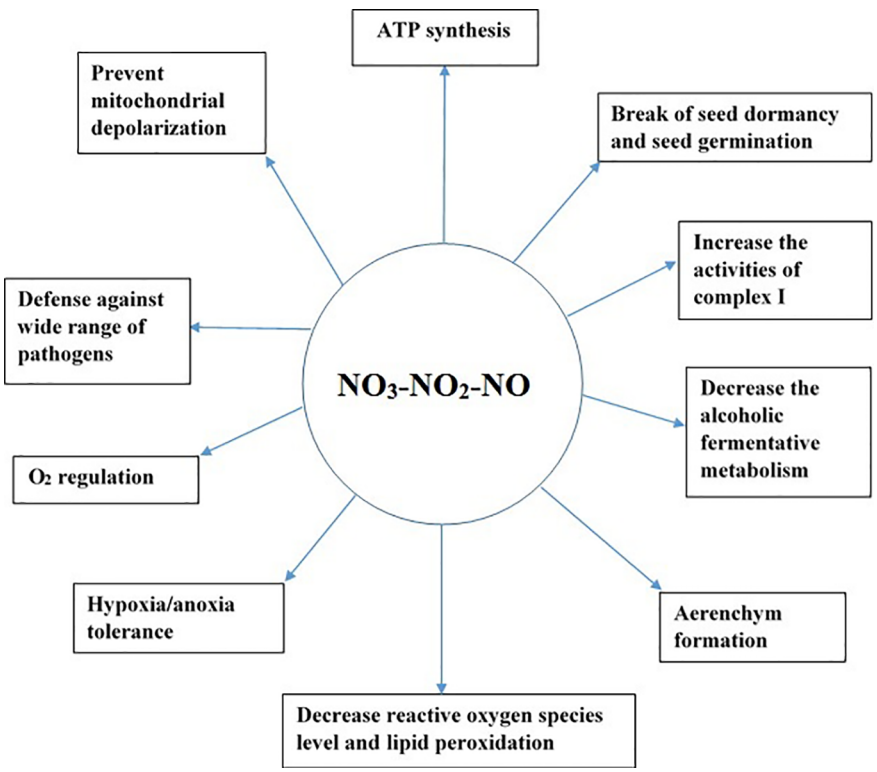


**FIGURE 2 |** Significance of the $NO_3$-$NO_2$-NO pathway in plants under hypoxic and anoxic conditions.

mitochondria to detoxify the high amount of NO formed, namely, oxidation of NO to $NO_2$ during normoxia (Cooper, 2002; Taylor and Moncada, 2010) and reduction of NO to $N_2O$ during hypoxia (Cooper, 2002). As both products of NO metabolism in the mitochondria, that is, $NO_2$ and $N_2O$, are non-toxic, their formation might play a protective role in the mitochondria. If hypoxia-induced NO production in cells is high, it can cause DNA fragmentation, leading to cell death; however, if NO is scavenged, it can reduce DNA fragmentation (Wany et al., 2017). Therefore, scavenging of NO is essential to protect cells from high NO toxicity. Phytoglobins are reported to scavenge NO in the cytosol (Igamberdiev et al., 2010). Additionally, purified mitochondria have been reported to scavenge exogenous NO (Gupta et al., 2005; de Oliveira et al., 2008; Wulff et al., 2009; Gupta and Kaiser, 2010). Furthermore, the addition of NADH as an electron donor increased NO scavenging by the mitochondria (Gupta et al., 2005; de Oliveira et al., 2008; Wulff et al., 2009; Gupta et al., 2016), indicating that the mitochondria have a protective mechanism to detoxify the excess NO formed. NADH might act as an electron donor to reduce cytochrome c oxidase, leading to an increase in NO scavenging in purified mitochondria. As discussed in our proposed pathway of $N_2O$ formation in the mitochondria, the conversion of NO to $N_2O$ by the reduced form of CcO might be the potential pathway regulating excessive NO formed under oxygen-limited conditions in the mitochondria. Mitochondria are not only a source of NO, but also an important sink and target of NO (Igamberdiev et al., 2014), and long-term exposure of mitochondria to NO can lead to the dysfunction of mitochondria (Brown and Borutaite, 2002). Although the $NO_3$-$NO_2$-NO pathway has several roles (**Figure 2**) in plants during hypoxia and anoxia, NO accumulation at higher level is toxic to cells (Brown and Borutaite, 2002). Therefore, $N_2O$ formation in the mitochondria *via* the $NO_3$-$NO_2$-NO pathway might be a strategy to protect cells and mitochondrial components from excessive NO formed under oxygen-limited conditions. Therefore, at molecular level, further research should focus on measuring NO and $N_2O$ from isolated mitochondria to obtain more insights on mitochondria's role in scavenging excess NO during hypoxia and anoxia.

## DO PLANT CELLS REDUCE $N_2O$ TO $N_2$?

The last two enzymes of the denitrification process, namely, nitric oxide reductase (NOR) and nitrous oxide reductase ($N_2OR$), merged to form CcO (Saraste and Castresana, 1994; Stanton et al., 2018). Moreover, the copper site in bacterial $N_2OR$ is similar to the CuA site in CcO (Kroneck, 2018). Many catalytic properties of CcO from denitrifying bacteria (*Paracoccus denitrificans*) and eukaryotic organisms are similar (Ludwig, 1987; Kadenbach et al., 1991). As eukaryotic mitochondrion is considered to be evolved from *P. denitrificans*, a denitrifying bacterium (John and Whatley, 1975), it may still possess rudimentary denitrification properties. Although during the evolution most of genes of the bacterium transferred to the nucleus, few remained in the mitochondrial DNA including genes of CcO (Kadenbach et al., 1991). Therefore, it may be possible that CcO of higher organisms might also possess similar properties like that of its ancestor, *P. denitrificans*. The significant negative relationship between $N_2O$ consumption and $CO_2$ respiration rates in plants and lichens (Machacova et al., 2017) suggests that mitochondria are the possible site of $N_2O$ consumption. This $N_2O$ consumption observed in these eukaryotes might be at the site of CcO, as this enzyme is formed from the last two enzymes of denitrification. There are also reports of emission of $^{15}N$-labeled $N_2$ from wheat crops supplied with $^{15}N$-labeled $NO_2$ (Vanecko and Varner, 1955), suggesting that under certain cell conditions, mitochondria may also metabolise $N_2O$ to $N_2$. However, to date, $N_2$ emission from plants is less reported. It may be due to the advanced systems of $O_2$ regulation in plants, and this might inhibit the complete process of denitrification. A recent study, which measured $N_2O$ and $N_2$ emission from soil–plant systems, showed that $N_2O$ and $N_2$ emitted by $NO_3$-rich soil–plant systems was three times higher than that by $NH_4$-supplemented soil–plant systems and bare soil (Senbayram et al., 2020), and this suggests that the possible role of $N_2O$ and $N_2$ production in plants. Further experiments at the molecular level (mitochondria) are needed to explore the reason for the significant negative relation between $N_2O$ emission and respiration rate in plants and lichen, as reported by Machacova et al. (2017).

## CONCLUSIONS

To cope with the problems of global warming and ozone layer depletion, a good understanding of $N_2O$ formation processes in various source is critical. Therefore, the $N_2O$ formation process in plants is a matter of concern. The reductive pathway of NO formation in the mitochondria along with further reduction of NO by ETC is a possible pathway of $N_2O$ formation in plants. Considering available evidence, we conclude that there is strong possibility that plant cells produce $N_2O$ in the mitochondria under hypoxic and anoxic conditions. The theory that plants are only a conduit for $N_2O$ produced by soil-inhabiting microorganisms might be an ambiguous explanation. The root zone may sense hypoxia and anoxia due to the soil environmental conditions, which may favour $N_2O$ formation in the root mitochondria. As some studies have shown that $N_2O$ emission from tree stems is higher than that from soils in natural habitats (Welch et al., 2019), the proposed pathway of $N_2O$ formation in plants might play a significant role in understanding N cycling in eukaryotic organisms and the global $N_2O$ budget. Furthermore, we have highlighted the reduction of NO to $N_2O$ in the mitochondria, and therefore, it would be valuable to reassess the role of mitochondrial ETC under both hypoxic and anoxic conditions. Although, $N_2O$ is a potent greenhouse gas ,its formation in the mitochondria might help to protect the integrity of the mitochondria and protect cells from the toxicity of NO accumulation during hypoxia.

## AUTHOR CONTRIBUTIONS

AT wrote the manuscript. CH supervised the whole work. CZ, BP, FB, and WD commented on the manuscript. All authors contributed to the article and approved the submitted version.

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