

Review Article

Biosensors Used for Quantification of Nitrates in Plants

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Nitrogen is essential for the plant because it is used for the production of chlorophyll, proteins, nucleic acids, amino acids, and other cellular compounds; nitrogen is available in two forms: ammonium and nitrate. Several tools have been used to quantify nitrates in plants such as the Kjeldahl method and Dumas combustion digestion; however, they are destructive and long time-consuming methods. To solve these disadvantages, methods such as selective electrodes, optical sensors, reflectometers, and images based sensors have been developed; nonetheless, all these techniques show interference when carrying out measurements. Currently, biosensors based on genetic constructions, based on the response of promoter gene fused to Gene Fluorescent Protein (GFP), are gaining popularity, because they improve the accuracy of measurements of nitrate by avoiding the interference of carriers ion, high salt conditions, and other factors. The present review shows the different methods to quantify the nitrogen in plants; later, a biosensors perspective is presented, mainly focused on biosensors based on organism genetically modified. The review presents a list of promoter and reporter genes that could be used to develop different kind of sensors, and a perspective of sensors to measure quantitatively the nitrogen is presented.

1. Introduction

Nitrogen is the most important nutrient in plants because it is required for chlorophyll production and other cellular compounds (proteins, nucleic acids, and amino acids) [1]. The nitrogen is disposable in two forms that are ready to be used by the plants: ammonium (NH_4^+) and nitrates (NO_3^-) [2]. Nitrate is absorbed in more quantities than ammonium [3]; however, if this absorbance is not balanced, in agreement to the requirements of the crop, it becomes in a factor that limits the normal growth of the plants [4] affecting drastically the crops production, mainly by the deficiency or toxicity of the nitrates [5].

The aforementioned reasons give the necessity of accomplishing a diagnostic of the nutrimental estate of the plants in a rapid, reliable, and precise way, in order to help the correct application of this nutrient. To achieve this, several tools and methodologies have been used [6]. However, the methods commonly employed, such as Kjeldahl Digestion and Dumas

combustion, are destructives and take long time for the analysis [7]. To avoid these disadvantages, rapid methods have been developed to determine the quantity of NO_3^- ; one of them is through nitrate-selective electrodes; however, this has presented interferences caused by the presence of other acceptor ions [8].

There are other methods based on optics principles to measure nitrates like radiometers, reflectometers, and sensors based on satellite images (dozel reflectance), leaf transmittance, and chlorophyll and fluorescence polyphenols [9]. These methods have high correlations between the optical parameters and nitrogen content of the plants and have the advantage of being nondestructive [7]; however, there are some drawbacks such as chlorophyll saturation, atmospheric interferences, and the high cost of the instruments.

Other technologies that could help to avoid the interferences presented by the optical sensors are the biosensors [10], principally those based on genetic constructions where a promoter sensitive to nitrate is fused to a Green Fluorescent

Protein (GFP) to measure, quantitatively, nitrates. This kind of sensors allows improving the precision of the measurements because there are no interferences produced by ions and external factors that could alter the estimations [10]; however, there are several factors that affect the fluorescence of the Green Fluorescent Protein (GFP), such as the presence of imidazole and high salinity [11]; nonetheless, the GFP does not have affinity with divalent cation, which is an advantage, unlike the other conventional methods used for quantifying nitrates [12]. The GFP is not an enzyme and results in being an excellent indicator because it does not interfere with the physiological mechanism of the cell [12]. The interval of PH of purification process is maintained generally between 7 and 8 as optimal for GFP [11].

The responses to this kind of biosensors are being employed due to its sensibility and versatility [13], being the sensitivity of the fluorescence proportional to the concentration of extracellular nitrate [10]; however, biosensors using GFP take long time to be prepared; developing this kind of devices takes a long time; so far, there are no suitable electronic devices for this task to be applied in crops.

2. Methods to Measure Nitrates

Several tools can be applied to measure nitrates in plants [6] and they have been reported to be suitable for achieving rapid analysis in plants with the goal of helping in the management of nitrates. Several methods for nitrogen quantification are presented.

2.1. Selective Electrodes of Ion-Nitrates. There are nitrate-selective electrodes in the market that are designed for being used in agriculture, allowing easy and fast measurements due to their portability. These characteristics allow this kind of devices to accomplish better nutritional programs. One of the most used sensors is the ion-selective electrode. These electrodes are based on ionophores, which are made by chemical compounds that are joined, selectively, to other ions of interest; these kinds of electrodes have been used for the analysis *in situ* due to their high selectivity. The sensors here presented requirement of an internal reference electrode and a solution, being the last drawback because it makes the maintenance of the device difficult [14]. Another problem presented in this kind of electrodes is the error that occurred when they are tested in high salinity solutions. Due to the presence of interference ions, such as chloride, the results could indicate the existence of these compounds, obtaining higher values compared with other methods [15]. The errors carried out by the high salinity solutions can be solved by adding silver sulphate; however, it results in being expensive. The most problematic situations are the high concentrations of chloride, bromide, and other compounds; when there are suspicions of having interferences, it is necessary to test the results in a reference laboratory. Calibration of the sensors could lead to better measurements [16].

Brambilla et al. [15] carried out a study about the validation (following the validation guide of assay methods of the 9 OAA (2008) and the guide EURACHEM (1998)) using three

distinct methods for determining the quantity of nitrates: ion-selective electrodes, nitracheck, and chromotropic acid were used to evaluate the parameters of veracity, precision, and uncertainty using several reference samples. The chromotropic methods result in being suitable for NO_3 analysis, because values of veracity were reached (veracity < 10% without significant references). Besides, the selective ion electrode method did not accomplish satisfactory results, because the required values of veracity and uncertainty were not reached; the reason of this was the high concentration of NO_3 that could be associated with high salinity content of the soil. The nitracheck method is not applicable for the determination of NO_3 due to its high limit of quantification, even though it have reached the veracity values (veracity < 10%).

2.2. Nitrate Strips. The strips of nitrate contain two reactive zones that change its color when they are exposed to a nitrate solution. The ions are reduced to nitrite: the nitrate ions are reduced to nitrites: the nitrite reacts with an aromatic amino acid and subsequently coupled to N-(1-naphthyl) ethylenediamine to form the color of the strip. The color intensity is proportional to the concentration of dissolved nitrates in the solution [6].

Niguyen and Angeles [17] carried out two experiments to determine the changes of the nitrate concentration in pineapple; the experiments were established to determine the samples protocols and to establish the critical values of NO_3 in sap related to NO_3 of the sap leaf and the vegetative yield. A Merkowuant nitrate was used, including NO_3 , and total N in tissue; this increases with levels of fertilization of N. The NO_3 of the sap increases during the 14:00 and 16:00 hours, as the level of fertilizer increases from 0 to 16 g. The plant increases the NO_3 and the total content of N of dry tissue. The NO_3 in sap was correlated with the total content of N, obtaining $R = 0.82$, and with the dry sap NO_3 , obtaining $r = 0.91$. The content of NO_3 varies from 397.33 to 418.21 and in dry tissue from 2282.76 ppm to 2490.96 ppm. Niguyen and Angeles [17] concluded that there will be need of more studies to establish the critical value of NO_3 in sap to validate the data and if it is possible to predict the necessities of N. A limitation of the strips of nitrate is its sensitivity to temperature that goes from a range of 6 to 30°C [6].

2.3. Chlorophyll Meters. The chlorophyll meter (SPAD) is a portable and easy-to-use instrument; it is not a destructive method and is functional to estimate, quickly, the nitrogen status of the crops; however, the chlorophyll meter is an instrument that measures indirectly the concentration of total nitrogen [18]. Several noninvasive methods have been developed in order to be applied in crops and to determine the nitrogen status of the plants. The majority of these methods calculate the nitrogen based on optical properties of the plants, which are affected by several factors such as water content, foliar senescence, diseases, vegetal nutrients, and nitrogen status within the plant [9].

The chlorophyll measurements do not interfere with the result over an excess of nitrates in vegetables, because there

are nitrates that immediately are accumulated in the leaves that are assimilated later; therefore, this accumulation is used as indicator of nitrogen instead of assimilated nitrogen [6].

Rorie et al. [18] correlated the chlorophyll values with the concentration of foliar nitrogen where a mean of 0.78 was obtained and a mean of 0.83 was obtained for all parts of the leaves. This high relation is in agreement with the previous work [19], whereas value of 0.84 for the correlation among a chlorophyll meter SPAD and the foliar concentration in leaves of corn was found. This leads us to think that chlorophyll meters could be used to fix the nitrogen deficiencies. Also, Liu et al. [20] accomplished studies with SPAD to estimate chlorophyll and the content of nitrogen in leaves, where it can be concluded, as the aforementioned work, that it is possible to estimate the chlorophyll content in the plants; however, even though there exists correlation among SPAD measurements and the nitrogen content during August ($p < 0.05$), in November, there were no significant correlations among nitrogen and SPAD measurements.

2.4. Optical Sensors Based on Fluorescent Dyes. Recently, research has been focused on optical sensors of nitrates based on the use of chloride of ammonium methyl tridecyl (TDMACI) as an ion selector; this compound is highly selective for chloride ion that is commonly in water and is used to avoid its interference [21].

Kim et al. [14] carried out studies for developing an optical sensor of nitrates based on disposable thin film. The sensor was fabricated by a nitrate-selective polymerase membrane on the surface of a polyester thin film. The membrane was composed of chloride of polyvinyl (PVC), fluorescent dye, and an ionophore selector. The intensity of fluorescence increased at the moment of contact with the nitrate solution. The response of the fluorescence of optical sensor was quantified through a commercial fluorescence meter. The optical sensor shows a lineal response through the four samples of ten repetitions. It was concluded that the concentrations of nitrates in nutritious solutions can be determined by optical measurements without any restrictions before the measurements; however, this kind of technologies is not able yet of disposing devices that integrate the chlorophyll quantification.

Dudwadkar et al. [22] use the method of anions interchange chromatography for quantifying nitrates through the detection of conductivity in suppression mode. A relative standard deviation of 5% was obtained and the limit of detection for the determination of nitrates was $0.02 \mu\text{g mL}^{-1}$. The method has high performance and analyzed 6 samples per hour. There were no interferences of impurities such as chloride and sulphate. The limit of detection for the determination of nitrate was 0.02 g mL^{-1} and the relative standard deviation was 5% for the general method.

2.5. Biosensors. The biosensors generally are defined as devices that consist of a bioreceptor and a mechanism of signal transduction able of producing a measurable signal in the presence of an analyte [23]. The term biosensor has been adopted by several technologies and can be qualitative and

quantitative and also can be devices that employ genetically modified organisms (GMOs) or physical devices. This kind of sensors recognizes, through the receptor, biochemical or physiological changes based on a system of signal transformation that is evaluated later [24] (see Figure 1). This term varies according to the applied approach and it could be qualitative or quantitative, physical devices, or transgenic cells, which eventually have a biological receptor that serves as signal recognizer detecting biochemical or physiological changes; eventually, a system of signal transformation is used to convert an event raised in a measurable signal [24].

Nowadays, the technology allows us to fuse an element with reporter gene to generate a cellular biosensor [10]. The proteins that emit fluorescence have been used as markers and as reporter genes to study the performance of proteins at cellular level and also used to measure, internally, the activity of a promoter in tissue [25]. When the gene of a protein is used as a transcription reporter, typically, a bioluminescent or fluorescent protein such as luciferase or the GFP [10], a cellular promoter, leads to the expression of the reporter gene, resulting in a measurable signal that reflects the expression of the promoter (see Figure 1). One of the bacteria that are used to be modified genetically to realize this gene expression is *Escherichia coli* (*E. coli*), where the aforementioned gene constructs have been tested; thus, the intensity of luminescence or fluorescence is directly proportional to the concentration of the analyte of interest (Figure 2).

There is great diversity of biosensors; however, highlight those based on immobilized enzymes located on the electrode which gives an electronic signal [26]. As mentioned above, proteins that emit fluorescence have been used as specific markers and as reporter genes for studying the performance of the proteins at cellular level [22]. When the gene is used as a reporter of transcription, a cellular promoter conducts to the expression of the reporter gene, resulting in a measurable signal that locally reflects the expression of the promoter. Commonly, this gene expression system is presented in the cell of *E. coli*.

3. Promoter Genes Sensible to Nitrates

There are a lot of promoter genes that can be used to build reporter bacteria for detecting some environmental change or several compounds, specifically, the detection of different forms of nitrogen presented in aquatic and terrestrial ecosystems [27]. Table 1 presents advantages and disadvantages of gene promoter that can be used as sensing element of any nitrogen source.

3.1. *glnA*. The *glnA* promoter that controls an operon of essential genes implied in the ammonium issues in Life Sciences Bacteriology, Parasitology, and Virology Acton assimilation (glutamine synthetase GS) [28] by amidation of glutamate for producing glutamine [29] has moderate activity under limiting conditions of nitrogen during exponential phase and is independent of nitrogen levels [28]; it is activated

TABLE 1: Advantages and disadvantages of gene promoters that can be used as a sensing element of any nitrogen source.

Gene	Enzyme	Advantages	Disadvantages
<i>glnA</i>	Synthetase glutamine	It could detect a wide range of concentration of ammonium in plants	A disadvantage is that it can respond to amino acids and other nitrogenous compounds
<i>nirA</i>	p-Hydroxyphenylpyruvate dioxygenase	It is induced in the presence of nitrate	It is repressed in the presence of ammonium
<i>GifA</i>	Synechocystis NtcA	It is induced in the presence of ammonium	It is repressed in the presence of combined nitrogen
<i>nitA</i>	Nitrilase	It is activated by combining a source of nitrogen ammonium nitrate	Change of the nitrogen source disrupts the growth and development
<i>pyeaR</i>	NsrR	It is activated in the presence of nitric oxide, nitrate, and nitrite	It is repressed by two repressors, Nar-regulated by nitrates and nitrites, and NsrR-regulated by nitric oxide

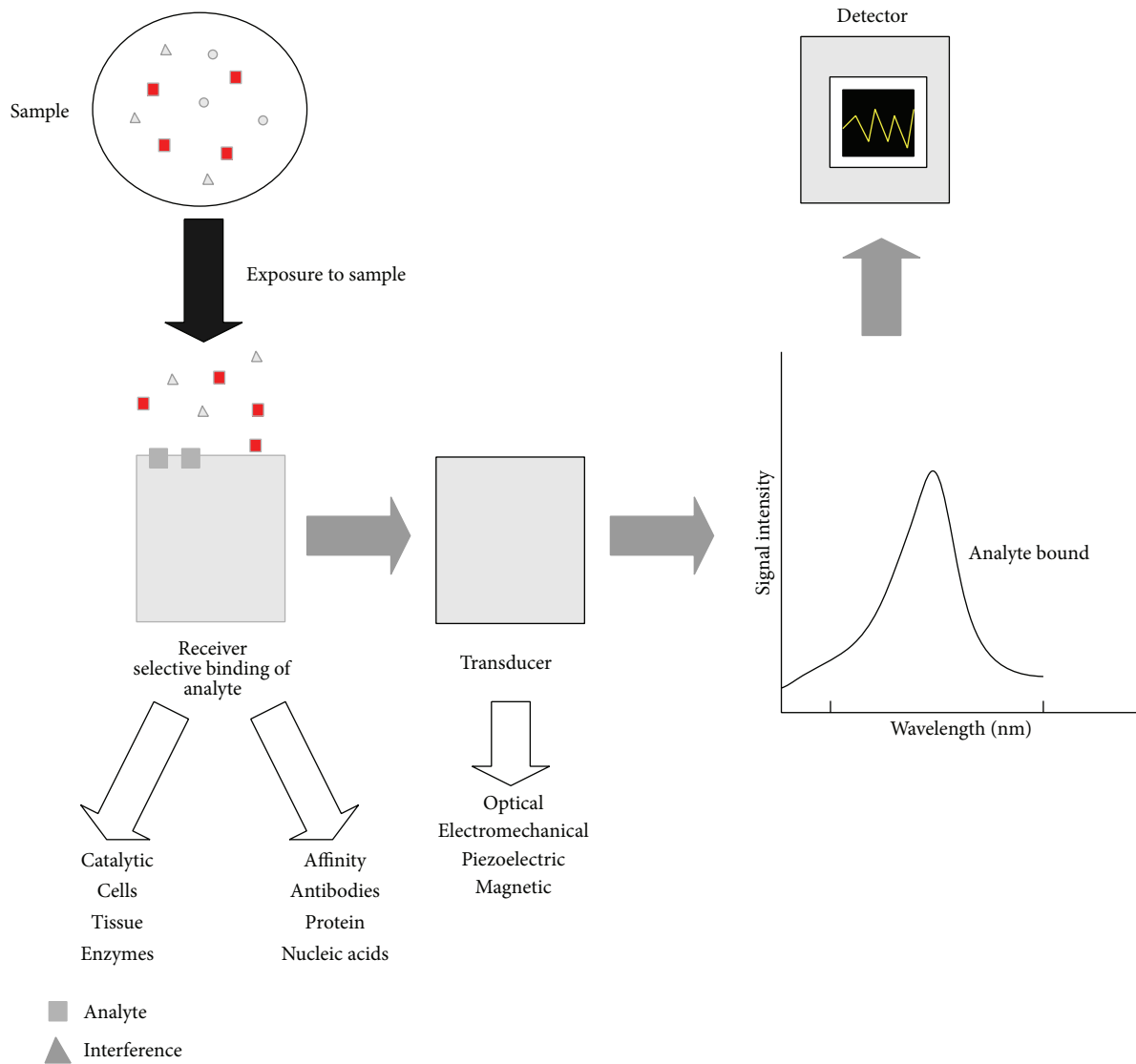


FIGURE 1: Schematic diagram of a biosensor. A bioreceptor is used to provide specific chemical recognition.

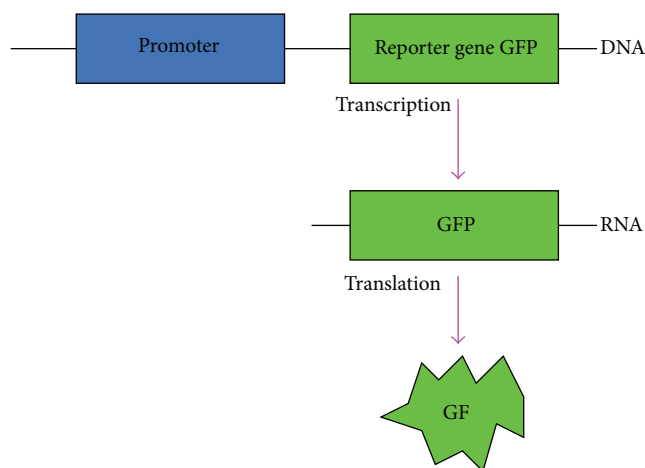


FIGURE 2: Reporter gene used to study a regulatory sequence.

in absence of different sources of nitrogen and responds linearly to the addition of known quantities of several nitrogen sources in the range of 50–500 mM NH_4^+ o NO_3^- [29].

3.2. *nirA*. The operon *nirA* is an intermediate for the assimilation of the nitrate reduction [30] and it is activated in presence of nitrate [28], in the absence of ammonium, and in the presence of nitrite [31]. It responds linearly in the gamma of 10–600 nM NH_4^+ [28]. When the nitrate is limited, the operon *nirA* becomes more sensible which leads to an increment of the activities of enzymes of nitrate assimilation [30]. Several positive actions of proteins needed for the expression of the operon *nirA* have been identified [31].

3.3. *gifA*. The promoter *gifA* is induced to be expressed in presence of ammonium, except in a medium that has different forms of nitrogen. GifA has a linear response going from 100 to 600 mM [28]. Bren et al. [28] carried out experiments where the expression of the gen *gifA* is in the strain NGIF and was tested in a medium with ammonium, a nitrate solution, and a solution composed of various forms of nitrogen and the levels of expression were low in solutions composed of several nitrogen forms and also in nitrate solutions; nonetheless, they were activated significantly from 6 to 24 hours in the ammonium medium [28].

3.4. *nitA*. The promoter of the gene nitrate reductase (*nitA*) performs as a molecular interrupter that allows turning on the expression of the transgenic species; this expression is activated combining a source of nitrogen of ammonium to nitrate [32] and these actions have allowed the development of new molecular tools that will be useful in the future [32].

3.5. *pyeaR*. *pyeaR* is found in the operon *yeaR/yoaG* of the bacteria *E. coli* and it is activated in the presence of the nitric oxide, nitrates, and nitrites. This promoter is found repressed by two repressor, *Nar* (regulated by nitrates and nitrites) and *NsrR* (regulated by the nitric oxide). *PyeaR* is not found active

in aerobic conditions; it can be used as a biosensor under various conditions [33].

4. Expression of Reporter Genes

The reporters' genes commonly used are *LacZ*, *luxCDABE*, and *gcp*; however, the development of the investigation has allowed the increase of the use of other fluorescent proteins such as *dsRed* (RFP) among others [34]. Table 2 shows the advantages and disadvantages of the reporter genes commonly used.

4.1. *Gfp*. The fluorescent green protein (GFP) is derived from *Aequorea victoria* and it is the most studied and used; there are a lot of variants that have been developed starting from the modifications done in GFP [22]. The expression of the GFP does not serve only as indicator; also, it is an indicator of the membrane integrity [35]. The promoter of interest controls the expression of the fluorescent green protein (GFP) [28].

The mFP is a dependent variant of the fluorescent green protein because it was identified in nonbioluminescence pathogenic bacteria (*Vibrio vulnificus* CKM-1), through the expression of the mBFP or the fusion of *Mbfp-msIC* evaluated in different subcellular compartments and tissues in three different plants. Dudwadkar et al. [22] showed that the protein mBFP is a reporter useful in plants which could be used as label to study the traffic of proteins and its localization.

A recombinant plasmid was introduced in the bacterium *E. coli* XLI-B and contains the reporter genes GFP y RFP, which were combined with the promoters *zraP* and *cusC*; it emits fluorescence in response to copper and zinc, resulting in an increasing of the fluorescence intensity [36]. The fluorescence emitted by RFP was low in cells exposed to zinc; however, brilliant red fluorescence was detected in cells exposed to copper; in the same way, the fluorescence of the GFP was low in the presence of zinc; nonetheless, brilliant green fluorescence under copper presence was detected. At the moment of combining these two elements (copper and zinc), the reporter genes were activated and showed yellow fluorescence. The construction of PCR GZl reporter detects, efficiently, low concentrations of 200 mM.

4.2. *lacZ*. The operon gene *LacZ* [37] is applied in cellular and molecular biology to solve genetic problems, recently, to attend emergent problems of genomics and proteomics, and it is used as a reporter gene due to its adaptation in the studies of promoter functions and the analysis of genetic expression. The expression of this protein could be used to detect the presence of substances in an environment, both *in vivo* and *in vitro*, based on the measurement of fluorescent products or colored derivatives [38].

4.3. *LuxCDABE*. This reporter gene obtained from other luminescent bacteria has been used due to its facility of being manipulated, sensibility, and its dynamic range of light measurement. This reporter gene produces a cellular signal

TABLE 2: Advantages and disadvantages of gene reporters that can be used as a sensing element of any nitrogen source.

Gene	Protein	Signal	Advantages	Disadvantages
<i>Gfp</i>	Green fluorescent protein	Green fluorescence	Multiple fluorophores No diffusion Does not affect the physiological mechanism of cell Compatible for translational fusions	Moderate sensitivity Temporal resolution Fluorescence microscopy
<i>LacZ</i>	β -galactosidase	Fluorescence, colored molecules	Can use inexpensive light microscope	Requires reagent
<i>dsRed</i>	Red fluorescent	Red fluorescence	Can use inexpensive light microscope	Moderate sensitivity
<i>CobA</i>	Uroporphyrinogen III methyltransferase	Red Fluorescence		Use fluorescence microscopy
<i>Luc</i>	Luciferase (insect)	Light	High sensitivity Linear across several orders of magnitude	Requires addition of coelenterazine
<i>LuxCDABE</i>	Bacterial luciferase	Light	High sensitivity and dynamic range	Moderate sensitivity and linearity
<i>LuxAB</i>	Bacterial luciferase	Light	Its high sensitivity and dynamic range, linear across	Requires addition of coelenterazine
<i>InaZ</i>	Ice nucleation protein	Ice	High sensitivity Linear across Excellent temporal resolution	Requires reagent

that helps to avoid the necessity of cellular disruption and enzymatic assays [39].

The development of new biosensors has been designed to transform an autotrophic bacterium *E. coli* through *gln* promoter (estandar, *gln*) and an operon reporter gene, to a biosensor *GlnLux* [39]. The biosensor *GlnLux* was tested in four different magnitudes, and linear measurements were obtained. For the calibration of the biosensor, a sensibility assay was done (signal:noise: ratio) and it was improved through the dilution of cultivation of the biosensor *GlnLux* to increase the availability of the *gln* per each cell. By employing these strategies, the biosensor *GlnLux* reaches a sensibility of 0.0001 $\mu\text{g}/\text{mL}$ (0.68 nM); this is a similar range as the obtained with the HPLC-MS/MS.

4.4. *LuxAB*. The system of bioluminescence contains five genes coming from the marine microorganism *Vibrio Fisheri* and was used by first time as expression gene in 1985 [40]. The *lux* genes were divided up in those to form a luciferase (*LuxAB*) and those required for synthesis of a large chain aldehyde (*LuxCDE*). The division of this system of five genes is much more effective than those using the complete operon *lux* (*luxCDE* + *luxAB* or simply *luxAB*); this was determined through the fusion of the gene *KatG* with the purpose of measuring precisely the potential of oxidative stress; thus, the divided *lux* system has a better signal than the fused complete operon; however, the system that contains only *luxAB* has higher signal than the others.

4.5. *InaZ*. The gene reporter codifies a protein of nucleation of ice of some bacterial species, such as *Pseudomonas syringae* that are proteins of external membrane that catalyze the ice

creation [41]. The dependent nucleation of nitrogen in two varieties of bacteria, *E. coli* DH5 and *E. herbicola* 299R, under anaerobic conditions that contain a fusion of the promoter gene *narG* with the reporter gene *inaZ* in a plasmid *pNarg-Ice* expresses activity of nucleation of ice that increases linearly with the increment of the concentration of nitrate in the interval that goes from 0.1 mM to 10 mM under anaerobic conditions [42].

4.6. *Luciferase*. Luciferase catalyzes a reaction (oxidation of benzothiazolyl-thiazide luciferin, magnesia, and oxygen) in order to create a reaction inside the range of 550 to 576 nm [43].

4.7. *Green Fluorescent Protein (GFP) as Reporter Gene in a Genetically Modified Bacterium*. The jellyfish *Aequorea victoria* formed by a protein that has the property of emitting green light has become in a useful tool to cellular biology [44]. The GFP is the protein that is formed by 238 amino acids [45]; this is the most studied and used, and there are several variants that have been developed by modifications of the GFP [22]. The promoter of interest controls the expression of the GFP [46].

Many proteins contain fluorescence proteins different of the sequence of the amino acid of the protein; the chromophore of the GFP is internally generated by a reaction of three amino acids [47]. The chromophore is formed by the spontaneous cyclization and the oxidation of the residues 65–67, corresponding to amino acids Ser 65, Tyr 66, and Gly 67 of the native protein and it is responsible for the emission of green light [44]. This chromophore presents two peaks of excitation and emission; if it is excited at 395 nm, it presents

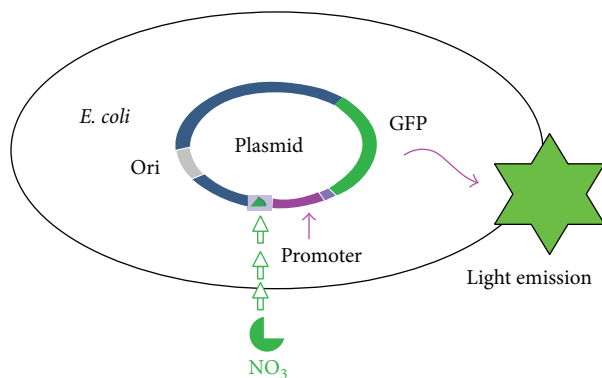


FIGURE 3: Basic principle of GFP microbial biosensors.

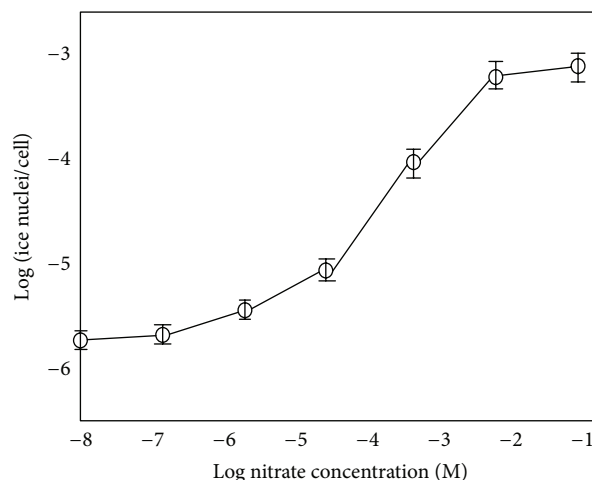
an emission peak of 508 nm; if it is excited at 475 nm, it presents a peak of emission of 503 nm [48]. To form the chromophore, a correct plication of the protein is necessary; if this plication is not achieved or makes an incorrect plication, the chromophore is not formed [47]. When the GFP reporter gene is used, it is attributed to a regulatory sequence of another gene (promoter); a promoter of interest is fused to coding region of the GFP that leads to the expression of the fluorescent protein resulting in a fluorescent [22] signal that temporally reflects the expression of a promoter *in vivo* [47].

The coding sequences of these fluorescent proteins in the plasmids are highly expressed in the system of the bacteria *E. coli* [11]; therefore, a correlation among fluorescence and the expression of the promoter of interest in bacteria genetically modified can be obtained. On the other hand, *E. coli* has been used as tool to detect large amounts of recombinant proteins [47]. Tsien [48] classifies the GFP as a passive application if it is used as marker fused to other proteins because it reflects the levels of expression of the protein or the subcellular location of this protein.

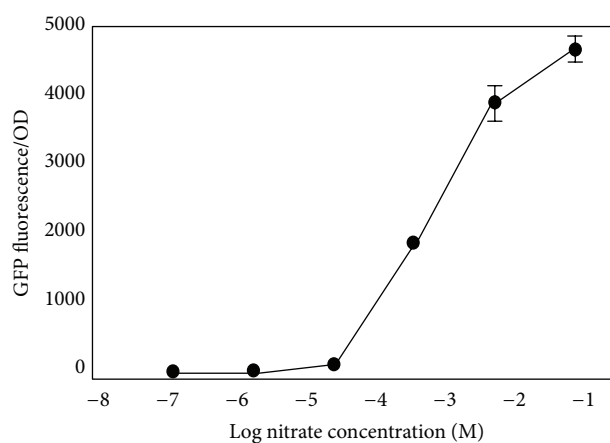
The biosensors based on fluorescence for the detection of nitrates have been reported. *E. coli* is transformed through a plasmid, where the promoter and the regulated regions of nitrates *Pnar* are fused to the GFP; the activity *Pnar-gfp* is measured in the range of concentrations of nitrates through the emission of the fluorescence of the GFP being the intensity of the fluorescence proportional to the concentration of extracellular nitrate without interference of other acceptors of electrons such as nitrites, dimethyl sulfoxide, trimethylamine n-oxide, and fumarate [10].

These gene constructs could be used to measure nitrates implants, because they present advantages with respect to the conventional sensors; because the GFP does not have affinity with divalent cations, the union and the elution of other proteins are not affected; the GFP results in an excellent indicator because it does not interfere with the physiological mechanism of the cell [12]; also, the interval of the PH of the purification process is maintained generally between 7 and 8 that is the optimal pH for the fluorescence of GFP; also, the high salinity conditions and the presence of imidazole are not factors that alter the stability or the fluorescence of GFP [11].

Figure 3 shows that the anatomy of a microbial biosensor for the determination of nitrates consists of transgenic



(a)



(b)

FIGURE 4: Ice nucleation activity of *E. coli* (pNice) and nitrate concentration (a). Fluorescence of cell cultures of *E. cloacae* (pNgfp) in minimal medium with different concentrations of nitrate. OD, optical density unit (b) [42].

E. coli-cell integrated in the genome as reporter gene GFP and sensible promoter gene nitrates [42]. Gene complex is transcribed into messenger RNA and then translated into a reporter protein that is ultimately responsible for generating a signal.

5. Development of Biosensors Nitrate Meters

These genetic transformations have been used to detect the availability of the nitrates in the ground around the roots. DeAngelis et al. report the development of two bacterial biosensors through *Enterobacter cloacae* (*E. cloacae*) and *E. coli* where the regulator promoter of nitrates in *E. coli* was fused to the promoterless ice nucleation (Inaz) and the reporter gene GFP through the plasmids *pNice* and *pNgfp*, respectively. The fluorescence of the GFP in *E. cloacae* through *pNgfp* plasmid was uniform to a given concentration of nitrates with a relation of 1000-fold when nitrate increased from 0 to 1 mM. The measurable induction of the nucleation of ice in *E. cloacae* through the plasmid *pNice* is given from

0.1 μM . The *inaZ*-based nitrate biosensor was responsive to nitrate concentrations about 10-fold lower (Figure 4) [42] which showed that the bacterial biosensors are useful to estimate the availability of nitrates in microbial niches in complex natural environments such as the rhizosphere.

There has been used the strain *Synechococcus* sp. FAM431 that contains the gene *glnN* fused to the reporter gene *luxAB*. The work presented by Muñoz-Martín et al. [29] accomplishes genetic constructions through the plasmids *Pbg2112*, *pB2110*, and gen promoter *nirA*. Muñoz-Martín et al. [29] have based these genetic transformations to realize a bioreporter bacterium. The development of these genetic transformations allows for studying the responses of the promoters *glnA*, *NIR*, and *gifA* to the exposition to different sources of the nitrogen in the strains *Nostoc* sp. PCC 7120 that express the region of the promoter of these genes fused to the reporter genes *luxCDABE* of *Xenorhabdus luminescens*.

Muñoz-Martín et al. [29] use the strain *Synechococcus elongatus* PCC 7942 that expresses the promoter *glnN* fused with the reporter genes *luxABAs* for detecting different forms of nitrogen in water; the results show that the promoter *glnN* fused with *luxAB* responds linearly in the range of 50–500 μM of combined nitrogen; however, with the operon *nirA*, the range of expression in the presence of nitrogen was 100 μM of NO_3^- . These differences between the ranges can be attributed to different forms of nitrogen presented in water; that is similar to the results of the strain *S. GLN*. Another bioreporter bacterium based on the promoter *glnAque* has the more reduced range of detection; it goes from 5 to 90 μM .

5.1. Considerations for the Development of Biosensors for Nitrates Determination

5.1.1. Immobilized Cell Systems. To develop biosensor of nitrates, it is necessary to provide systems for immobilizing cells; this helps to avoid problems associated with the growth of cell population [49]. Cell immobilization refers to confined and located cells, retaining their catalytic activities. These cells can be immobilized permanently or temporarily in order to be used repeatedly in various chemical processes [50].

The considered criteria for conditions of detention are determined to build strong granules, high substrate transfer rates, and low rates of cell leakage [51]. The advantages of biotech systems based on immobilized cells are their ease of handling high density cells, improvement of the control in continuous systems, and the recovery of biomass for reuse [50].

One of the methods used to immobilize cells is the preparation of macroporous solids using porous materials synthesized by unidirectional freezing and subsequent lyophilization [50]. Hydrogel materials have been used as an immobilization matrix cell. The high viscosity pre-gelled solutions used in these applications decrease cell viability due to the shear forces required for mixing the cells with these solutions, which is associated with decreasing the molecular weight of polymer used for the gel capacity; it enables gelation. Immobilization of cells with alginate hydrogels allows for increased cell viability of 40% to 70% compared

with the use of polymer chains to form high molecular weight gels [52].

To immobilize cells of *E. coli* (concentrated aqueous suspension of *E. coli* bacteria) within a polymeric matrix, calcium alginate and glucose have been used by freeze-drying technique; the resulting matrix that is a porous matrix of polyvinyl alcohol bacteria is retained in such a way that is able to detect specific molecules from an environment [50].

5.1.2. Fluorescence Measurement System. Considering the development of biosensors for nitrates, common *E. coli* is modified to make these bacteria specifically express genes (i.e., luminescence or fluorescence produced by the expressed gene) in response to nitrates presence in the medium. Luminescence or fluorescence intensity of the fluorescent protein is directly proportional to the nitrates concentration; thus, it will require fluorescence detection systems that allow for making a correlation between nitrates and the emitted fluorescence [10], since fluorescence sensors are an attractive target for the development of a new generation of detection systems for nitrates since the methodologies based on fluorescence are more sensitive and easier to apply than current technologies [53].

Fluorescence is a phenomenon that occurs in various biological processes and systems where molecules absorb and emit radiation in a spectral range of 475 and 508 nanometers (nm) [54]. This phenomenon has become a powerful tool in investigations in studies at the molecular level and dynamics of living systems [55]. If a fluorescent sample with adequate power is irradiated, the molecules absorb such energy; this energy enables movement of a valence electron from the ground state GS_0 (being an electronic singlet where electrons have opposite spin and net twist is 0 a higher energy level) to an excited state ES_n [56]. After the emission of the photon, the molecule returns to its ground state [57]. Photon emission accompanying the passage at vibrational level is a spontaneous process called fluorescence [55].

Several studies have enabled the development of new small and cheap fluorimeters by using light emitting diodes (LEDs) that turn blue sign [54] where results were found favorable.

Given these considerations, using systems in bacterial fluorescence measurement based biosensors builder complementary DNA (reporter gene (GFP) and a promoter gene sensitive to nitrates in an expression vector in prokaryotic cells (*E. coli*) [58]), is possible to develop a system quantizer fluorescence to determine the amount of nitrates in a solution.

6. Perspectives

There are biosensors based on gene constructions that indicate the concentration of nitrates; this occurs when a complementary DNA and the promoter gene are inserted in prokaryotic cells [58]; this quantification is accomplished through fluorescence correlation. The response to these kind of biosensors is being commonly employed due to its sensibility and versatility [13]; the intensity of fluorescence is

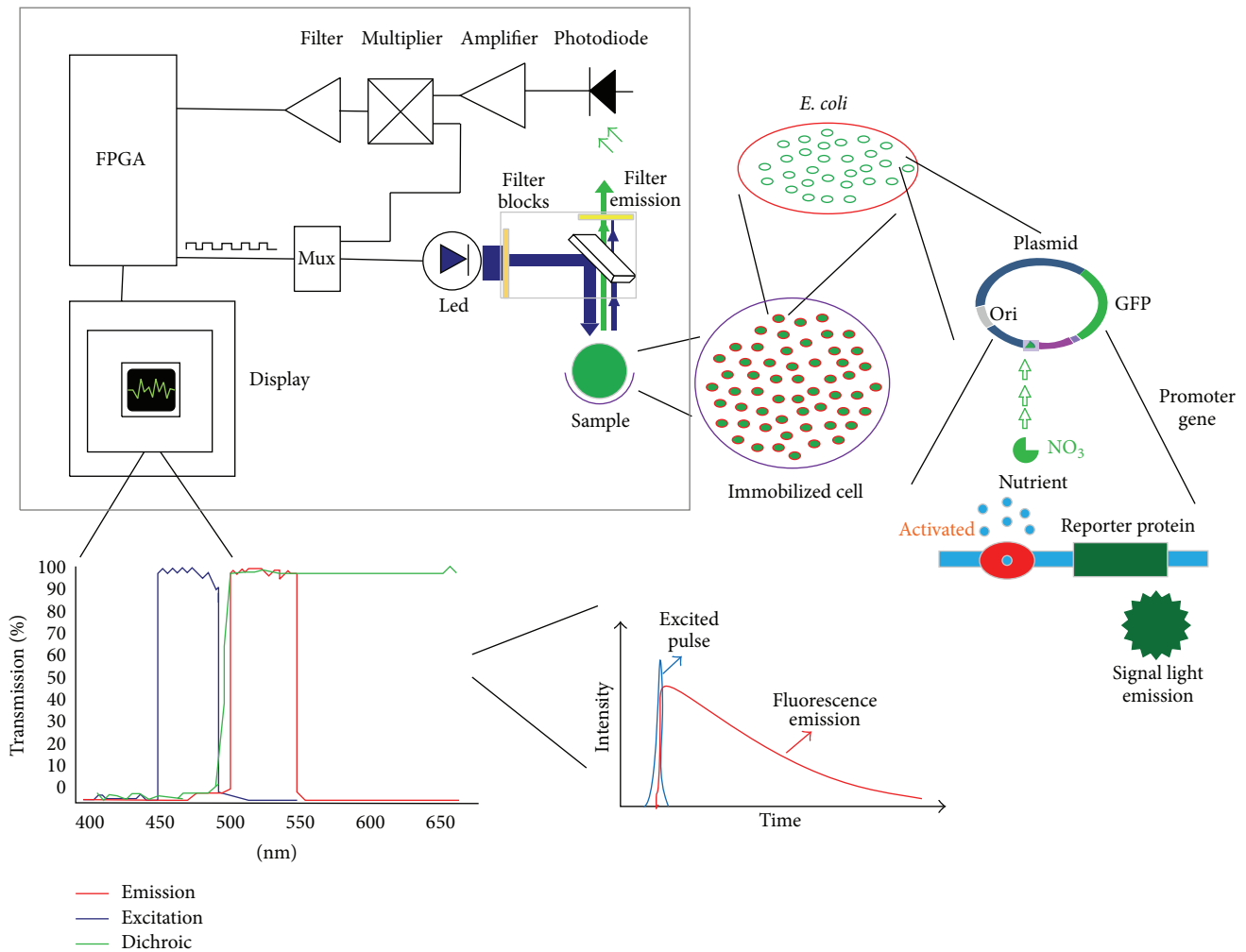


FIGURE 5: Integration of the necessary elements for the design of biosensors for nitrates.

proportional to the concentration of the extracellular nitrates [10].

This review provides necessary information and gives guidelines for the integration of biological and electronic systems for developing biosensors for nitrates detection and quantification using a fluorescence detection system (Figure 5). The latter could be composed of a processing unit; in fact, there are currently biological processes units using processing units [59]; one of them are the FPGA (Field Programmable Gate Arrays). FPGAs have been used for developing processing systems for correlation spectrometry in radio astronomy [60]. The use of these devices in the area of biosystems is becoming popular mainly due to their high reconfigurability and SoC (System on a Chip Solutions). The methodology proposed here is the following: the processing unit (FPGA) sends pulses to LEDs with emission spectrum of 475 nm, directing the light through the emission filter that only allows the passage of emission spectrum generated by the LED [54]; often, a dichroic mirror directs the light from the LED to the sample; the genetically modified bacteria with the GFP gene are excited by the incidence of the light and fluoresces in the range of 508 nm; an excitation

filter of 508 nm only allows the passage of the light in the range of 508 nm. The fluorescence emission will be assessed by a photodiode which converts the number of photons received in a corresponding number of electrons [61]. The electrons will be moved to a conduction band generating a photo-current; this photo-current is converted to a voltage by signal conditioning system and, later, this signal is sent to an analog-digital converter. Finally, the processing unit receives this signal in order to process it [62]. Quantification process could be possible if promoters are sensible to other compounds, and the reporter genes are identified, and by combining both, the researchers could obtain a quantitative relation of the emitted fluorescence and the quantity of the compound under analysis. To use these biosensors, it is necessary to perform a standardized protocol and design a strategy involving cell structure by a reporter and receiver nitrates gene [10]; according to the review, these biosensors could be disposable by using encapsulated agarose pellets or sodium alginate as cell immobilization tool in order to avoid the problems of increase in bacterial population or population decay [49]. These cell structures (bacteria) will be replaced when a new measurement is made; one

that promises portability equipment has the ability to store the bacterial biosensors with the sample being required to measure. Finally, the use of the fluorescence emitted by GFP produced by the open reading frame coupled to a responsive promoter nitrates allows estimation of their concentration in solution using a mathematical model involving nitrate concentration and intensity of fluorescence; this model could be obtained using a mathematical regression model.

Unlike other methods, there are works where the interferences by ions and external factors alter the measurements, and these disadvantages could be avoided if biosensors based on gene constructions are used [10]. However, gene construction based biosensor takes more time to be prepared because there are no devices based on gene constructions that measure the nitrates in versatile form [13]. This kind of devices could be developed commonly because the principle of fluorescence is known. Nonetheless, based on this principle, it would be possible that the researchers could develop more of these kind of sensors not only for nitrate.

7. Conclusions

The development of biosensors based on constructions genetically modified that could indicate, quantitatively, the quantity of nitrogen has been a breakthrough and step by step is becoming a robust tool with high potential for being used in crops because of the advantages of not interfering with the physiological mechanism of the cells [12], and the problems, such as those generated by carriers ions, the union, and elution of proteins, are avoided by using this kind of biosensors. Also, high salinity conditions and the presence of imidazole are no factors that interfere with the measurements of this kind of biosensors [11]; therefore, it is thought that genetically modified biosensors are the future of measurement systems, because apparently by using this approach, great diversity of sensors could be developed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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