POLYOR SAS TECHNICAL DOCUMENT 4

INTEGRATED FERTILISER MANAGEMENT AND AZB™ as a case study (IAF)

A*zotobacterial biofertilization*, AZB™, involves the application of free-living nitrogen fixing bacteria such as *Azotobacter* spp. to soil-borne crop residues. Non-*Fabaceae* field-crops such as spring and winter cereals, rapeseed, sunflower and grain-corn, benefit from AZB, more so since it incites residue retention. Because of its ability to bring to the soil-plant environment exogenous nitrogen without compromising yield by diverting photosynthates, azotobacterial fertilization can also sustainably enhance AgroNum™'s N-fertilizer response curves. To do so, AZB must increase both grain & grain-nitrogen yields, along with NUE. This will impact N-fertilizer recommendations. In the context of *integrated fertilizer management*, a series of AZB agronomic field trials 2001 → 2016 are summarized herein.

A synopsis of Polyor's IP applicable to AgroNum™ and ifm

AZB™ : *Azotobacterial fertilisation* of winter wheat & rapeseed for integrated fertilizer management

EP2845906
EP2942621
EP3120680
EP3335536
EP3417691
EP3479671
EP3537157
EP3679779
etc., etc.

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NOTE

The following pdf is a technical working document provided by Polyor SAS as a preamble to further exchange & discussion. These technical descriptions are loosely adapted from the corresponding patent documents. For published documents, links to European patent office's databases are provided via www.polyor.fr's IP (intellectual property) thumbnail.

As mere working documents, these synopses are not for further publication. For the sake of coherence, the information & data are those appearing in the *original* patent documents. This said, and as part of Polyor's continuing R&D, many have since been updated, finetuned & further validated.

Polyor's AgroNum™ approach to *integrated fertilizer management* is new and disruptive. AgroNum™ may at times seem iconoclastic, if not irreverent, to some. To get a clearer understanding of the stakes at hand, feel free to contact Polyor SAS (www.polyor.fr) directly.

Azotobacterial fertilization - Introduction

Polyor SAS (Polyor) is an *intellectual property holding company* with its own *in silico industrial research* capacity dedicated to *azotobacterial fertilization* (AZB™) and more generally *integrated fertilizer management*, ifm, or more specifically herein - IAF (*integrated azotobacterial fertilization*) as it applies to AZB. For instance, Polyor's IP portfolio could eventually be licensed to an industrial firm manufacturing N, P, S & cationic fertilizers most likely to benefit from AZB & IAF. If need be, sublicenses can be given to suppliers specialized in the fabrication of the aforesaid AZB constituents.

As of today, AZB is a sprayable liquid formulation of azotobacteria applied to cellulosic *soil-borne crop residues* prior to their burial and the seeding winter field crops such as cereals and rapeseed. AZB's constituents, though relatively simple and easily manufactured by firms in the sector, are combined, metered, formulated, and packaged according to a series of innovative concepts patented for the most part by Polyor.

AZB was first developed in the USSR early XXth century and in North America until the 2nd WW[1]. AZB was thereafter abandoned in Europe & North America in favor of plant x microbe interactions and a sort of scientific cold war. AZB is also to some extent used in India and SE Asia[2] given the paucity and relative high cost of NP-fertilizers. It is not however always clear if these azotobacterial PGPR[3] are primarily applied to seeds, soil or crop residues, the supply of the latter – given low yields, likely being a limiting factor for AZB in these areas.

Europe has thus not been very open to AZB due to an institutionalized prejudice against non-symbiotic N-fixation, and more particularly the magnitude of its contribution to the yield potential of field crops such as wheat.[4]

A previous commercial *bio-activator* approach to AZB development insistently advocated the systematic reduction of N-fertilization as a tradeoff for the cost of purchase and application of this technology. Again, it soon became evident that this simple and de facto reduction of N-fertilizer application rates was counter-productive. Unfortunately, this reduction as a selling point trumped any other agronomic and commercial consideration.

The efficacy of AZB has been assayed over the past 20 years via a series of in vitro, in situ and in silico Polyor experimentations. Still, AZB problematic for logistical reasons concerning the handling and conservation of such bacterial inoculants. Paradoxically, this represents a development opportunity for Polyor and licensees.

Context and opportunity

Sustainable agriculture, soil conservation, and the abatement of CO_2 and N_2O emissions and non-point source pollution have unleashed the commercial development of a plethora of bio-stimulation and bio-fertilization concepts[5].

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[1] Emerson 1917. Soil inoculation with azotobacter. *Retrospective Theses and Dissertations.* Paper 14737 http://lib.dr.iastate.edu/rtd/14737 ; Sullivan 1938. Some factors influencing nitrogen fixation by Azotobacter. *Retrospective Theses and Dissertations*. 15203. http://lib.dr.iastate.edu/rtd/15203 , etc. etc.

[2] Available references on demand ; Martin et Brown 1938 ; Baltensperger et al. 1978 ; Yadav et al. 2003 ; Kumar et Ahlawat 2004, Senapati et al 2004, Kader et al. 2000 et 2002, Mahboob et Asghar 2002, Idris 2003, etc.

[3] Plant growth promoting (rhizo)bacteria

[4] P-P. Claude and L. Fillion. 2004. The effect of bacterial inoculation of soil-borne grain-corn crop residues on the yield and quality of winter wheat in France. Agrosol . 15 (1): 23-29.

[5] http://www.biostimulants.fr/ ; http://www.biofertilisants.fr/

For the most part these are either *plant x microbe interactions* such as rhizobia, mycorrhiza and PGPR[6]s and/or (supposedly) *activators* (sic) of soil microflora[7].

Plant-microbe interactions are scientifically well poised but will inevitably divert the flow of photosynthates thus reducing yield potential because of this un-called for competition[8]. *Soil activators* and their like are conceptually even more shifty and vague[9]; soil health and activity are said to be increased, which – in echo, should improve soil structure and build organic matter. These interactions and biostimulants can supposedly reduce N-fertilizer needs and/or increase yields and reduce recommended $N_{fertilizer}$ rates. It is unclear how this drift away from optimum yields can necessarily increase in $NUE_{I/O}$ and CO_2/N_2O abatement.

Polyor's AZB approach is an alternative to such biostimulatnts and biofertilizers. AZB integrates rather than excludes or circumvents N, P, S and cationic nutritionals. AZB is neither plant x microbe interaction (biofertilizer) or biostimulant, but rather seeks to exploit in situ *soil borne crop residues* as a carbon and energy source for non-symbiotic azotobacteria. In doing so, field-crop yield potential is maintained or increased since photosynthates are *not* diverted from the phloem to the symbiotes. The soil microflora is *not* targeted since only the *residusphere* need be impacted which is technically sounder.

The redirecting of N, P, S and cationic fertilizers from soil and roots to less predestined plant organs such as leaves and seeds represented at the time a major paradigm shift. Though the applications of liquid fertilizers to leaves and to/near seeds (banded, localized, starter, etc.) were longed recognized as feasible, their widespread use lingered. Today, the redirecting of these same fertilizer salts to soil borne crop residue as an alternative to leaves and seeds is also at a crossroad. AZB is thus a major market opportunity for those involved in the manufacturing and agronomics of fertilizers and nutritionals.

AZB is in many aspects like foliar fertilization since AZB is designed as a liquid formulation sprayed directly onto soil borne crop residues prior to their soil incorporation in the fall. For instance and as depicted in **Figure 1**, soil-borne crop residues are AZB-treated post-harvest (Figure 1 ; A) prior to the seeding of a winter field crop (wheat in this case). AZB's azotobacteria will « fix » atmospheric dinitrogen non-symbiotically *and* (over)immobilize residual soil N. This N will be released early spring and contribute to the winter field crop's N-nutrition. In the course of the growing season, the crop's standing biomass can also be AZB-treated using otherwise conventional *nutritionals* (Figure 1; B), advantageously as a result of in-season yield monitoring (Figure 1 ; C). These nutritionals will not only fill the current crop's NPKS needs but also enrich this standing biomass precursor of the next season's soil-borne crop residues. Post-harvest, these now soil-borne crop residues can be « re-treated » with AZB prior to the fall seeding of another field or cover/catch crop. Etc., etc.

Examples of how existing (integrated) fertilizer technologies and knowhow can be applied to Polyor's *integrated azotobacterial fertilization* (IAF) are presented herein. The further experimental and agronomic development of these inventions will ensure that Polyor's licensees procures all the exclusive knowhow, licensing and leadership in the field of AZB → IAF fertilisation.

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[6] Plant growth promoting rhizobacteria

[7] Faessel et al. 2014. Rapport final | Produits de stimulation en agriculture visant à améliorer les fonctionnalités biologiques des sols et des plantes. http://www.rittmo.com/

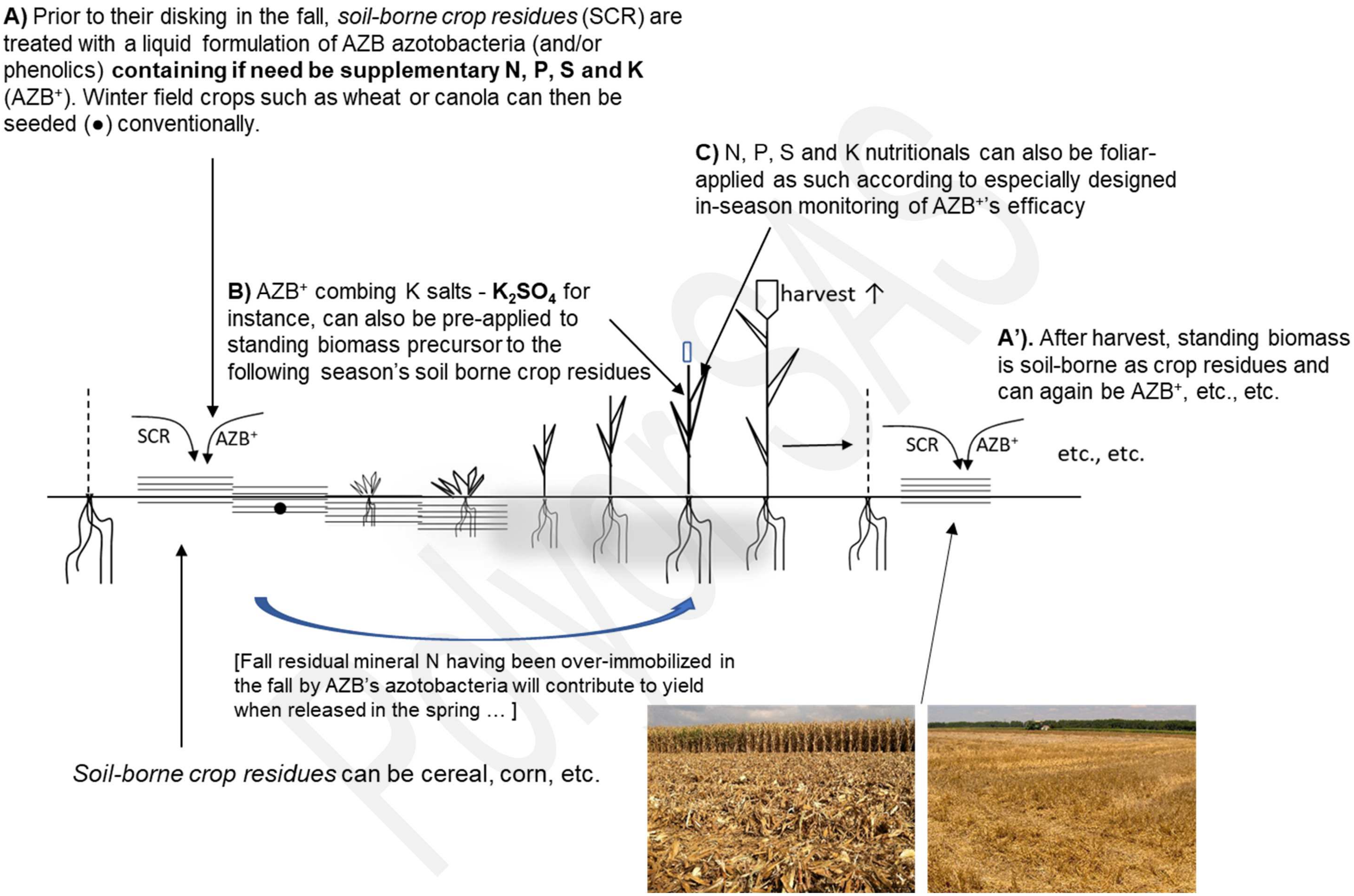
[8] Lynch et al. 2004. Rhizoeconomics: Carbon costs of phosphorus acquisition. Plant and Soil (2005) 269: 45–56

[9] Some examples DéchaumActiv™ : https://www.viavegetale.com/produits-viavegetale/dechaumactiv/; FreeN™ : https://gaiago.eu/micro-organisme/, SolActiv™ : https://www.youtube.com/watch?v=DZxZa1OdgAI / http://www.biofertilisants.fr/.

Figure 1 (following page)**:** Existing N, P, S (eg. thiosulfates) and cations (K, Mg and Ca) preparations can thus be integrated to AZB at three (3) different instances in the course of azotobacterial fertilization (AZB) of winter field crops;

A. directly onto the soil borne crop residues, after the harvest of the preceding cereal, corn or rapeseed crops producing these residues;
B. by treating the standing biomass of the aforesaid preceding crops (and defoliating potatoes as well) producing these residues before their harvest;
C. as foliar nutritional complements onto the AZB-treated winter field crop according to an in-season diagnosis of its yield potential.

The A approach is the most intuitive and assayed as of today. For logistical and practical reasons, the B approach to AZB is interesting since other foliar treatments such as fungicides, growth regulators and nutritionals can be applied simultaneously to the preceding crop producing the crop residues to be returned to the soil surface. This B approach is not intuitive and requires that dosages, compatibility and application dates – of K_2SO_4 for instance, be substantially modified (increased) ; this is presently the object of some of Polyor's *in silico* industrial research and IP. Finally, the aforesaid liquid fertilizers cans also be applied as such to the AZB-treated winter field crop. This third (C approach) is of course independent of azotobacterial fertilization (AZB) per se but will nevertheless be more sought after given that in-season yield potential monitoring is beneficial to AZB; several Polyor patents concern such in-season monitoring of AZB-affected yield potential.



A) Prior to their disking in the fall, *soil-borne crop residues* (SCR) are treated with a liquid formulation of AZB azotobacteria (and/or phenolics) **containing if need be supplementary N, P, S and K** (AZB^+). Winter field crops such as wheat or canola can then be seeded (●) conventionally.

C) N, P, S and K nutritionals can also be foliar-applied as such according to especially designed in-season monitoring of AZB^+'s efficacy

B) AZB^+ combing K salts - **K_2SO_4** for instance, can also be pre-applied to standing biomass precursor to the following season's soil borne crop residues

A'). After harvest, standing biomass is soil-borne as crop residues and can again be AZB^+, etc., etc.

[Fall residual mineral N having been over-immobilized in the fall by AZB's azotobacteria will contribute to yield when released in the spring …]

Soil-borne crop residues can be cereal, corn, etc.

Figure 1 : Azotobacterial fertilization (AZB) as a cropping practice

Agronomic potential of AZB ; its limits and how to overcome them ?

The measurement of non-symbiotic N-fixation in situ has been tried in the past, in Australia[10] and the American mid-west (**Table 1**), for instance. This American research was in response to the putative development of azotobacterial fertilization in the USSR where low-yield / low-input cropping scenarios early XXth century were conducive to the development of fertilization technologies that do away with mineral N-fertilizers. In western Europe however, where yield potential is higher and N-fertilizer abundant, non-symbiotic N-fixation was considered marginal at best, irrelevant most often. This R&D was to all extent and purposes abandoned on the eve of the 2nd WW for various reasons.

Table 1 : Some historical from the American mid-west

- 1917 : Soil inoculation with *Azotobacter* / Paul Emerson (*Iowa State College)*
- 1920 : Studies on *Azotobacater chroococcum* Beij. / Augusto Bonazzi (Ohio Agricultural Experiment Station)
- 1931 : Effects of N and P compounds *Azotobacter* and the fixation of N / L. Garnett Thompson Jr. (*Iowa State College)*
- 1932 : Winogradsky culture method for determining certain soil deficiencies / Arthur Wesley Young (*Iowa State College)*
- 1937 : Factors influencing the occurrence of *Azotobacter* in Iowa soils / William Paxman Martin (*Iowa State College)*
- 1938 : Some factors influencing nitrogen fixation by *Azotobacter* / John Loraine Sullivan (*Iowa State College)*

Interestingly, these data reveal that in most instance the agronomic potential strictly in terms of ΔN equivalent to $N_{fertilizer}$ is in fact in the whereabouts of 20 to 25 uN, i.e. non negligible agronomically. Rothamstead's long-term winter wheat plots in place since 1843 corroborate this putative net non-symbiotic N-fixation contribution[11].

As depicted in **Figure 2**, the contribution of non-symbiotic N-fixation attributable to AZB is in fact multifaceted. The contribution of non-symbiotic N-fixation will not only bring mineral-N into the soil-root-residue system, but also de facto lower the rCN (C-to-N ratio) of the residusphere; stochiometrically, this will hasten the mineralisation of the residue's N fraction which would have otherwise remained recalcitrant.

Residue-borne azotobacteria will also transiently (supra- or over-) immobilize residual mineral-N in the fall and retain it up until early spring when it will be released and made available to the blooming winter crop. Fall residual Nm is thus transformed by AZB into a N-input increasing the overall efficacy of AZB (cf. Figure 1 herein). PGPR-like effects attributable to the developing roots coming into contact with residue-borne azotobacteria are also likely but have not been documented; this contribution is probably marginal anyway.

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[10] Roper et al. 1983. Field Measurements of Nitrogenase Activity in Soils Amended with Wheat Straw. Aust. J. Agric. Res., 1983, 34, 725-39

[11] http://www.era.rothamsted.ac.uk/Broadbalk/bbk_open_access_yields

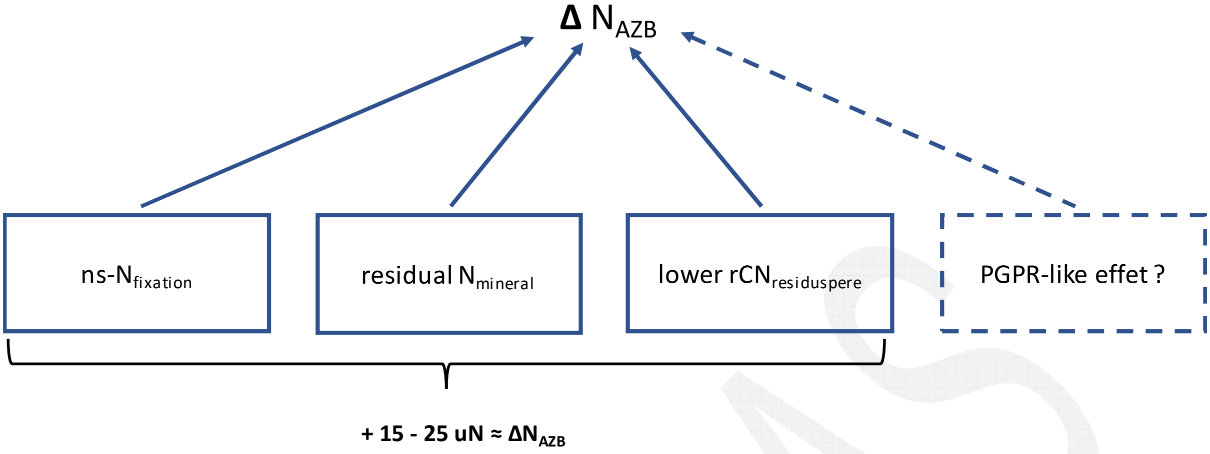
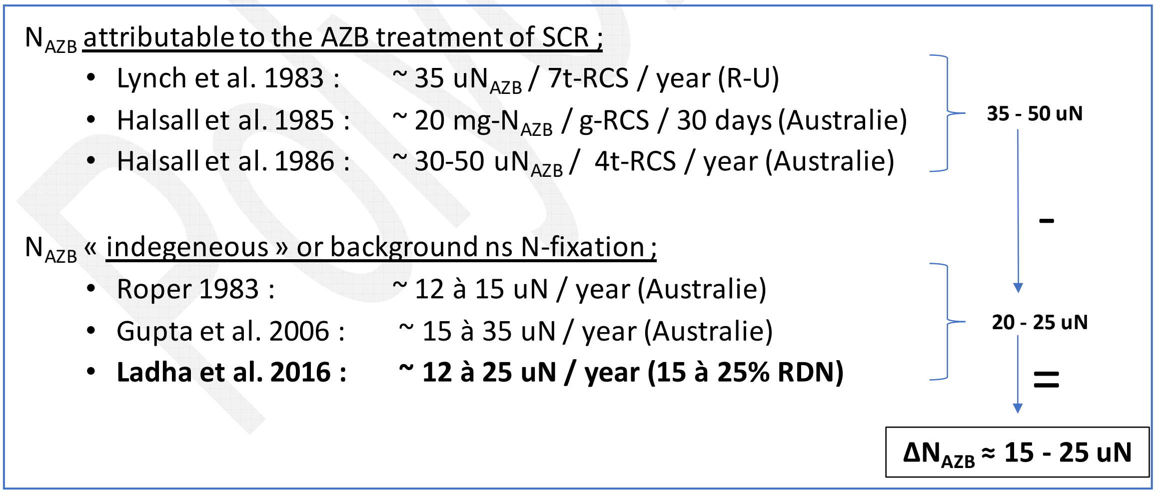


Figure 2 : The various sources of agronomic N attributable to AZB (ΔN_{AZB})

Thus, the net AZB contribution in terms of available Nm is in the whereabouts of 20uN. Quantitatively (**Table 2**), this N-contribution can be ascertained using (relatively rare) published data. To do so, we must distinguish between the contribution of azotobacteria on or near the crop residue, and that of the background or indigenous azotobacteria. The potential N benefit – strictly in terms of $N_{fertilizer}$, is in the whereabouts of 20uN; the field trial data corroborated this a priori crude estimate. However, Polyor firmly believes that the in situ non-symbiotic N-fixation is not marginal and can be bolstered using AZB.

Table 2 : AZB inocula as compared to indigenous azotobacterial N-fixation[12]



N_{AZB} attributable to the AZB treatment of SCR ;

- Lynch et al. 1983 : ~ 35 uN_{AZB} / 7t-RCS / year (R-U)
- Halsall et al. 1985 : ~ 20 mg-N_{AZB} / g-RCS / 30 days (Australie)
- Halsall et al. 1986 : ~ 30-50 uN_{AZB} / 4t-RCS / year (Australie)

35 - 50 uN

-

N_{AZB} « indegeneous » or background ns N-fixation ;

- Roper 1983 : ~ 12 à 15 uN / year (Australie)
- Gupta et al. 2006 : ~ 15 à 35 uN / year (Australie)
- **Ladha et al. 2016 : ~ 12 à 25 uN / year (15 à 25% RDN)**

20 - 25 uN

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ΔN_{AZB} ≈ 15 - 25 uN

It is important to note that the efficacy/benefits of AZB as portrayed by Polyor should not only be assessed in terms of N-fertilizer equivalents, but rather in terms of increased yield and NUE. For instance, see below the 2011 to 2015 field data obtained with « classical » AZB.

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[12] **Lynch and Harper 1983**. Straw as a Substrate for Cooperative Nitrogen Fixation. Journal of General Microbiology (1983), 129, 251-253. / **Halsall et Gibson. 1985**. Cellulose Decomposition and Associated Nitrogen Fixation by Mixed Cultures of Cellulomonas gelida and Azospirillum Species or Bacillus macerans. Appl. Environ. Microbiol. 50(4): 1021-1026 / **Halsall et Gibson 1986**. Comparison of Two Cellulomonas Strains and Their Interaction with Azospirillum brasilense in Degradation of Wheat Straw and Associated Nitrogen Fixation. Appl. Environ. Microbiol. 51(4):855-861 / **Gupta et al. 2016**. Potential for non-symbiotic N2-fixation in different agroecological zones of southern Australia. Australian Journal of Soil Research, 2006, 44, 343–354 / **Ladha et al. 2016**. Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. Nature Scientific Reports | 6:19355 | DOI: 10.1038/srep19355

Field trails (**Table 3**) harvested in France from 2011 et 2013 to assess the efficacy of AZB inocula on winter field crops such as wheat and rapeseed (alias “canola”), were carried out. More than 200 paired-plot comparisons involving nearly 400 observations of grain and protein yield and NUE were analyzed; more than half of these comparisons included the reduction (rationing) of the recommended $N_{fertilizer}$ rate (alias dX).

Table 3 : Agronomic benefits attributable to AZB inocula (see Annexe for further data).

2011-13 dX	**control**	**AZB**	**eAZB (AZB/control)**	**tTest**		**No. comparison**
rate $N_{fertilizer}$ (uN)	182	182	1,00	0,50	ns	64
% grain humidity	15,5	14,7	0,95	0,00	**	56
% grain protein	12,0	11,9	1,00	0,38	ns	61
yield (Qx grain/ha)	**52,3**	**56,1**	**1,07**	0,02	*	62
yield (kg protein/ha)	**628**	**677**	**1,08**	0,03	*	60
NUE (kg protein/uN)	**4,19**	**4,53**	**1,08**	0,03	*	60
SW (kg/hl)	71,66	73,67	1,03	0,00	**	61
TGW (g/1000)	38,98	41,34	1,06	0,00	**	61

2011-13 dX - uN	**Control**	**AZB**	**eAZB (AZB/control)**	**tTest**		**No. comparison**
rate $N_{fertilizer}$ (uN)	193	173	0,89	0,00	**	105
% grain humidity	11,8	11,9	1,01	0,35	ns	102
% grain protein	11,8	11,4	0,97	0,04	*	99
yield (Qx grain/ha)	**66,6**	**69,0**	**1,04**	0,03	*	102
yield (kg protein/ha)	**776**	**783**	**1,01**	0,36	ns	99
NUE (kg protein/uN)	**5,01**	**5,49**	**1,10**	0,00	**	99
SW (kg/hl)	76,87	77,05	1,00	0,27	ns	98
TGW (g/1000)	41,03	42,40	1,03	0,02	*	80

AZB inocula increased grain and protein yields by approximately 8% ; NUE was increased by up to 10%. These increases were equivalent to a 60 to 70€ gross benefit. However, the rationing of dX was counterproductive because of a transient supra-immobilization of soil mineral nitrogen (Nm). Polyor has since proposed formulations and application strategies aimed at overcoming and exploiting this supra-immobilization of Nm. These data were corroborated in situ in 2014 and 2015 for winter wheat and rapeseed (canola) ; see Annex.

However, and as hinted in the aforesaid, the efficacy/benefits of AZB as proposed by Polyor is best appreciated in terms of increased (protein) yields and NUE, not in terms $N_{fertilizer}$ economy (rate reduction). Misconceptions as to the goals, benefits and merits of AZB hampered its development in the past. Still, AZB inoculation as it stands today can be said to have the following outcome;

- 3 to 400 kg-grain/ha
- 50^+ kg-protein/ha
- 65^+ € gross margin /ha
- 8 to 10% increase in $NUE_{protein}$
- Soil organic matter build-up
- Improved use efficiency of fall post-harvest residual mineral N

The agronomics of AZB as of today were nevertheless seen as problematic. For instance, as mentioned herein AZB will not de facto reduce N-fertilizer needs since yield potential tends to increase over time. NUE will however be improved which is most important in agro-ecologically. In addition, and given the absence of symbioses, AZB azotobacteria need to be indigenous (i.e. site and/or soil specific). As of today, this *endogenicity* was assured using "custom made" site-specific inoculant. The logistical limitations of this approach soon became apparent and need to be overcome. An alternative is a Polyor patent-pending approach (**EP2728353**) for the creation of biogeographically specific azotobacterial consortia which do away with the need for a site-by-site production and reintroduction of indigenous azotobacteria. This however requires in fine multiple registrations.

Polyor concluded that the development of AZB → IAF would be hasten if the logistics surrounding the production, conservation, handling, and application of such (live!) bacterial inoculant could be circumvented. A three-pronged industrial research and IP strategy was developed to that effect.

Polyor has since devised an *integrated* approach for the optimization of AZB (*integrated azotobacterial fertilization*, or IAF) consisting of a chemotaxic approach to AZB advantageously combing **complex N, P, S and cationic nutritionals**. IAF also couples the N-fertilizer rate recommendations to the potential efficacy of AZB as diagnosed using especially designed indices and yield monitoring technologies. IAF is thus the future of AZB (**EP3335536)** and will be discussed explicitly. This integrative approach to AZB involving Existing nutritionals is described in the accompanying **Document II** of the present series; "*Agronomics and optimization of AZB"*.

Agronomics of AZB ; an integrative approach to optimization

Since the full potential of AZB has not yet been deployed, the object of Polyor's industrial research and intellectual property is to bring about the transition of AZB to the more performing IAF (*integrated azotobacterial fertilization*). Given logistic and agronomic limitations, the agronomics of AZB as developed to date were revised to included mineral and simple organics (phenolics) as substitutes for inoculants. AZB can now be more easily complemented with various N, P, S and cationic nutritionals.

Circumventing the ill-advised reduction in $N_{fertilization}$ rates is one aspect of this AZB → IAF transition and involves the development of a series of *indices* of AZB's efficacy that can be bundled (aggregated) and serve to calibrate $N_{fertilisation}$ rates especially catered to AZB (**iAZB ; EP1719651.7**). When aggregated, these indices allow for the recalibration of $N_{fertiliser}$ rates, these rates being more precise *because* they cater especially to AZB.

The greater use of foliar applied N, P and S liquid fertilizers is another means to this AZB → IAF transition. This more intensive use of foliar fertilization has led to the development of innovative in-season monitoring approaches to crop yield potential monitoring as affected by AZB (**pAZB**). Finally, in-season yield monitoring protocols (**pAZB**) assessing the need for additional foliar applied nutritionals given the increased yield potential attributable to AZB have been proposed and patented by Polyor.

More importantly, a third aspect of this AZB → IAF transition involves the use of various N, P, S and cationic nutritionals along with AZB as a sprayable liquid formulation. P and S requirements of crop residue-borne azotobacteria can be met using P and S nutritionals produced and/or commercialized by Polyor's licensees. Triazones for instance can also be used to – paradoxically, lessen the expression of N_2O emitting periplasmic nitrate reductases. Finally, the cationic portion of many of these nutritionals will partially neutralize the cellulosic crop residue's CEC which tends to otherwise repulse azotobacteria. These concepts increasing AZB's efficacy they are referred to **eAZB** concepts.

This three-pronged approach for the optimization of AZB and its transition (evolution) towards IAF is depicted in **Figure 1**, whereas the various technologies are partially listed in **Table 1**. Again, these three (3) phases (stages; **eAZB**, **iAZB** and **pAZB**) bring about the optimization of AZB → IAF as portrayed in **EP3335536.** As we will see, various existing nutritionals are involved mainly in **eAZB** as explicited by some Polyor concepts such as CTX, DEN, THS, HRP, etc.

Some of the notions underlying these techniques as patented by Polyor will be briefly explicated below.

As mentioned in **Figure 2**, The transition from AZB to IAF is a function of these three sets of concepts (**eAZB**, iAZB and pAZB); existing nutritionals being mainly involved in improving **eAZB**. AZB will soon be widespread; the firm having a head-start and – more importantly, a technical edge will dominate this market sector. The yield and NUE increases attributable to this transition to IAF will procure the licensee with a competitive advantage [b – a] and ensure its leadership. Given this leadership, the user of IAF will prefer IAF over the more "classical" (i.e. inocula) AZB approach to azotobacterial fertilization.

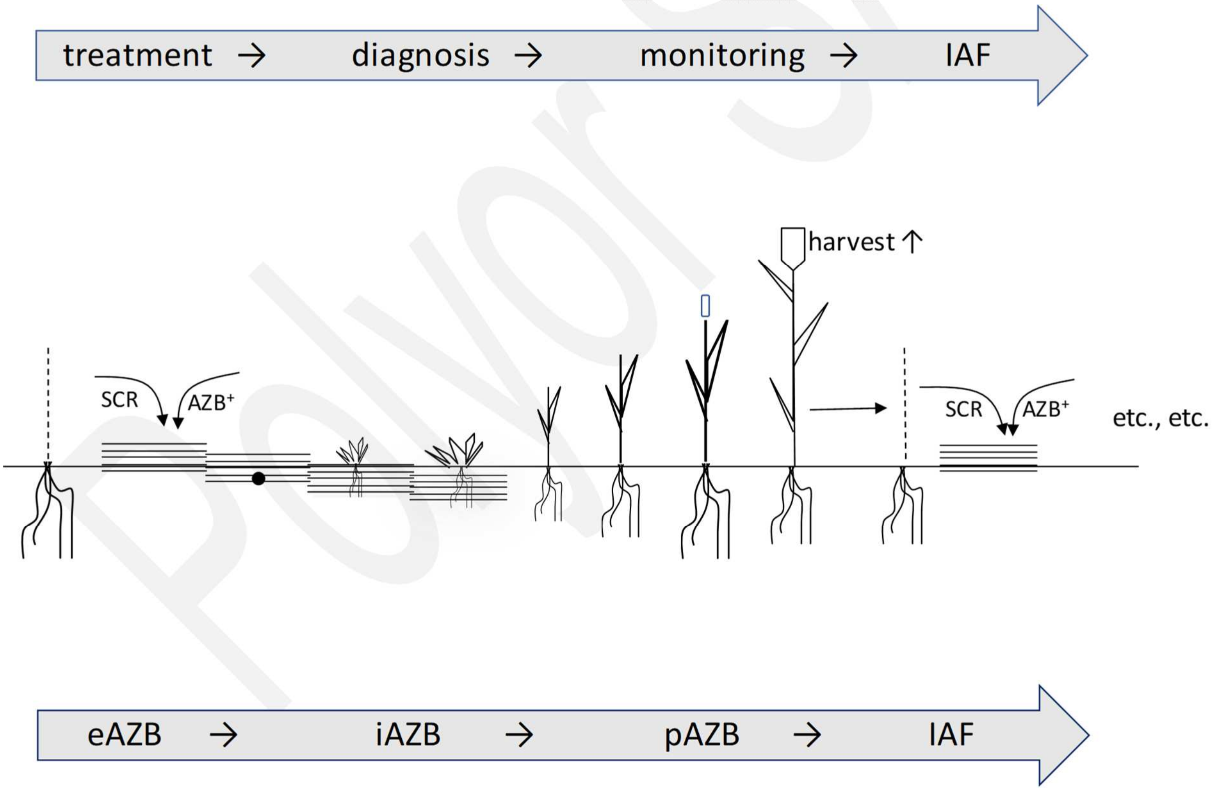

Figure 1 : Polyor's approach for AZB's transition to IAF

Table 1: Some Polyor concepts[13] application to IAF (… and existing nutritionals)

eAZB → azotobacterial ***treatment*** of soil borne and standing crop residues

- **eCTX** : EP3417691 - AZOTOBACTERIAL FERTILIZATION WITHOUT INOCULATION OF SCR
- **eDEN** : (FR1900152) - AZOTOBACTERIAL FERTILIZATION WITH MITIGATED N_2O EMISSIONS
- **eHRP** : (FR1901429) – CROP RESIDUES ENRICHED IN PHOSPHORUS AND PHENOLICS
- **eTHS** : (FR1901431) - APPLICATION OF THIOSULFATES IN AZOTOBACTERIAL FERTILIZATION
- ETC., ETC.

iAZB → quantitative appraisal and ***diagnosis*** of the efficacy (potential) of eAZB

- iREC : EP2845906 - METHOD FOR ASSESSING THE CARBON EFFICIENCY OF SOIL BACTERIA
- iRES : EP2730926 - DIAGNOSISING THE pH RESILIENCE OF SOIL BACTERIAL POPULATIONS
- iDAM : EP2942621 - DIAGNOSIS OF THE DIAZOTROPHIC STATE OF ARABLE SOIL
- iRNN : EP18203377.9 - MINERAL-N AS AN INDICATOR OF AZB EFFICACY
- ETC., ETC.

pAZB → in-season ***monitoring*** of the crop nutritional status

- pNNI : EP2915420 - DIAGNOSIS OF THE PHYSIOLOGICAL STATE OF AGRONOMIC CROPS
- pNNS : EP2942622 - METHOD FOR DETERMINING CRITICAL NITROGEN CONTENTS OF CROPS
- pNNO: EP2671443 - MULTIVARIATE DIAGNOSIS OF NUTRITIONAL STATE OF FIELD CROPS
- *[pNNA : FR______ - IN-SEASON INDICATOR OF CROP YIELD POTENTIAL (… in the works)]*
- ETC., ETC.

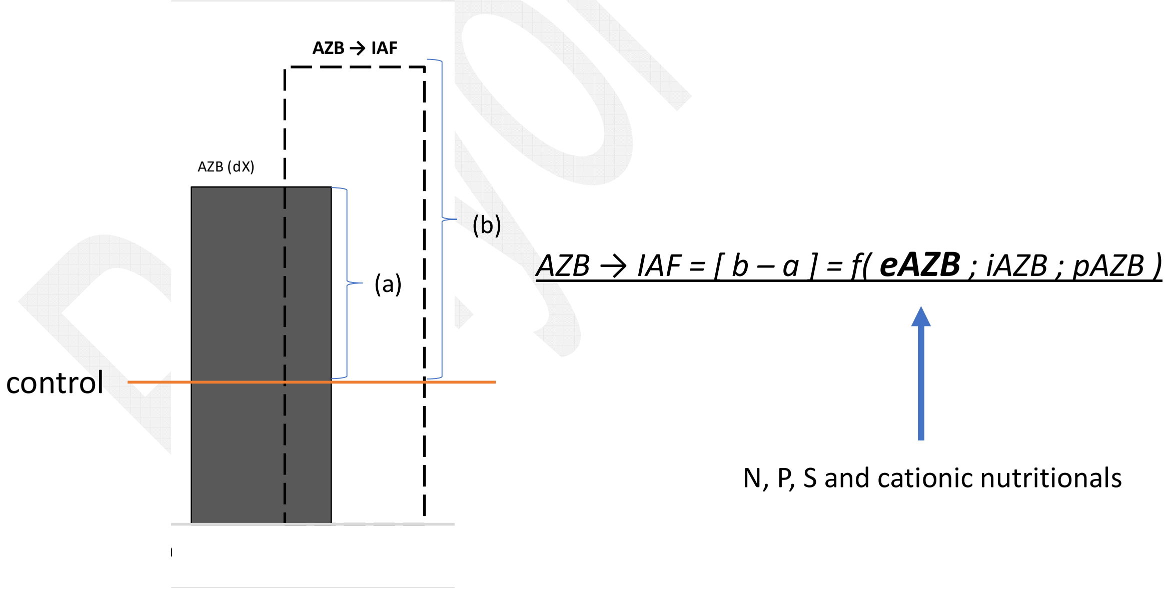

Figure 2 : The [b – a] IAF differential as a function of eAZB, iAZB and pAZB

One of the pivotal concept (CTX - **EP3417691**; **Figure 6**) will effectively do away with the imperative use of (azoto)bacterial inocula via the innovative use (dosage) of chemotaxic agents that favour the migration over a few mm of a minimal proportion of indigenous azotobacteria from the *pedosphere* to the *residusphere*.

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[13] The three (3) concept-patents **DEN**, **HRP** and **THS** will most likely be combined in a single patent application this fall 2019. See herein, footnote page 9 of 14.

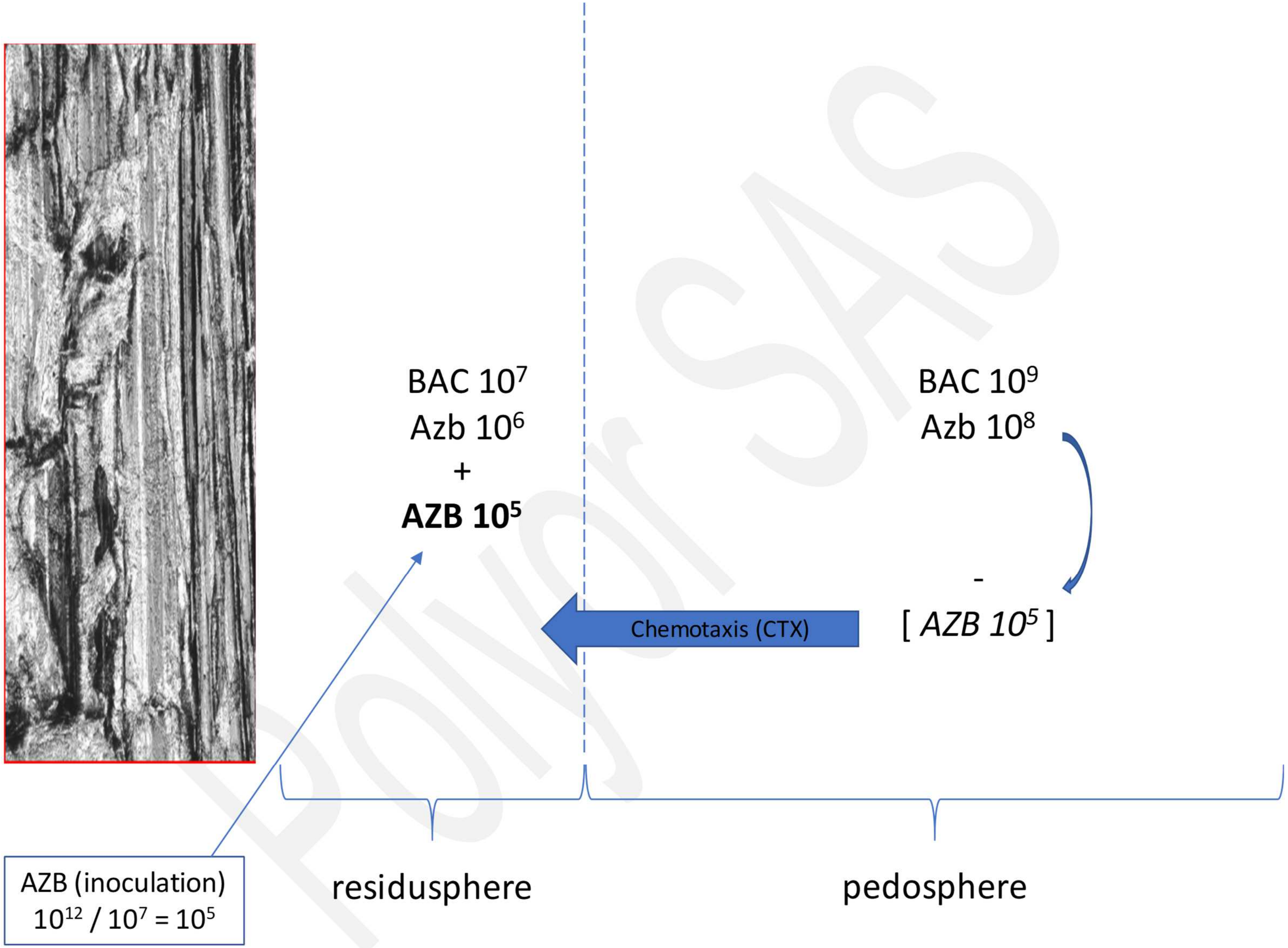

Figure 6 : Schematic representation of Polyor's CTX concept as an alternative to AZB inocula

A BRIEF HISTORY OF AZB

AZB was first developed by Polyor (Pierre-Philippe CLAUDE) in 1997 in France during a post-doc at ENSAT (École nationale supérieure d'agronomie de Toulouse). [Nb. Conceptually, AZB was a spin-off from PPC's previous work with *in situ* soil decontamination using endogenous soil bacteria.]

1997

Initial R&D (FR2833016) and greenhouse trials of various types of azotobacteria applied to cellulosic crop residues ; data available on demand (NDA).

2000

Various formulated and packaged AZB preparations were tried in the field with in-season monitoring of winter field crop; data available on demand (NDA).

2004

Tentative AZB two-strain consortia (AZB$_{3186}$) in view of registration was tried in situ (field) across France

2008

Endogenous AZB preparations were reintroduced on a site-by-site basis as an effective alternative to the registration of AZB$_{3186}$

2016

Reconfiguration of Polyor's patent portfolio given AZB's evolution towards IAF (*Integrated azotobacterial fertilization*)

2019

IAF integrates almost twenty years of in vitro, in situ and in silico experimentation. A series of European patents enabling the further experimental and commercial development of IAF could be licensed to an industrial firm manufacturing N, P, S fertilizers and cationic nutritionals.

AZB will in the future thus be accomplished without inoculation, thus greatly simplifying its production, sourcing, logistics and field application. More importantly, the aforesaid complementary use of existing nutritionals for the optimization of eAZB is greatly facilitated by use of chemotaxic agents such as certain phenolic acids in lieu of AZB inoculants.

The functioning of CTX (**Figure 6**) implies that the bacterial (BAC) contingent of the soil is approximately $10^{e}9$/g-soil. At least 1/10 (a conservative estimate) of this contingent are azotobacteria (Azb). Given the mass of crop residues relative to that of the topsoil, one percent (1%) of the soil's azotobacteria are immediately in contact with (or within 3-4 mm of) the buried crop residues, or approximately $10^{e}6$ / g-crop residue. Surprisingly, though the AZB-azotobacteria inoculated onto these crop residues represent only a fraction (1/10) of these indigenous azotobacteria (Azb), this has proven to be agronomically consequent.

CTX will allow azotobacteria receptive to triggering by phenolics to migrate these few mm from the *pedosphere* (or « bulk soil ») to the *residusphere* ; this microscale migration implies that 1/1000 (0,1%) of pedosphere azotobacteria migrate. This feasibility of such microsale migration has been demonstrated using a recognized model of bacterial chemotaxis.

Agronomic trials (2011-2015)

Field trails harvested in France in 2011, 2012 and 2013 to assess the efficacy of AZB on winter field crops such as wheat, were carried out. More than 200 paired-plot comparisons involving nearly 400 observations of grain (**Figure 1**) and protein yield (**Figure 2**) and NUE (**Figure 3**) were analyzed; more than half of these comparisons included the reduction (rationing) of the recommended Nf dose (aka dX). AZB increased grain and protein yields by approximately 5 to 8% ; NUE was increased by up to 10 percent on average.

These increases were equivalent to a 60 to 70€ economic gross benefit. However, the rationing of dX by some 20uN was on average counterproductive, most likely because of a transient supra-immobilization of soil mineral nitrogen (Nm). Polyor has since proposed several formulations and application strategies aimed at overcoming and exploiting this supra-immobilization of Nm. Business and technical opportunities arise *because* AZB (i) does not respond well to reductions in Nf, (ii) inoculated or ambient azotobacteria are advantageously endogenous and adapted to local *agro-pedo-climates* (APC) and more specifically (iii) gluco-reactive. IAF (integrated azotobacterial fertilization) ensures that these conditions are met.

Figure 2 reveals that despite its attractiveness, the conventional (intuitive?) Nf = dX – 20 uN AZB scenario is - on average, counterproductive in terms protein yield (grain yield x [% grain protein / 100]) given a relatively small (~ 0,5%) but significant reduction in grain protein content (data not shown). This tampering with dX to all extent and purposes annihilates the agronomic benefit in terms of increased grain yield (Figure C) and is thus not sponsored by Polyor. Instead, it is Polyor's IAF (integrated azotobacterial fertilization) approach to AZB with its (i) improved treatment and application techniques integrating some existing nutritionals traditionally applied to foliage (**eAZB**), (ii) more precise quantification of AZB efficacy (**iAZB**) used to fine-tune dX and (iii) novel in-season crop nutritional status monitoring (**pAZB**) that will ultimately optimize AZB.

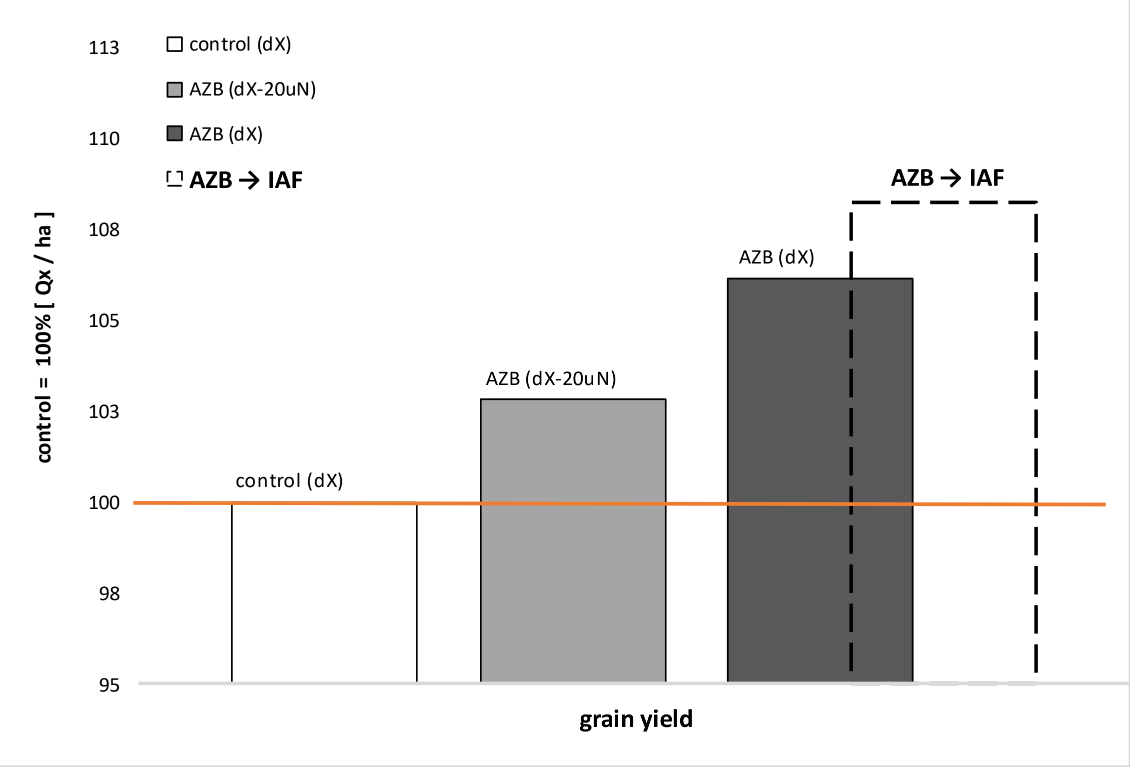

Figure 1 : The agronomic consequence of AZB → IAF[14].

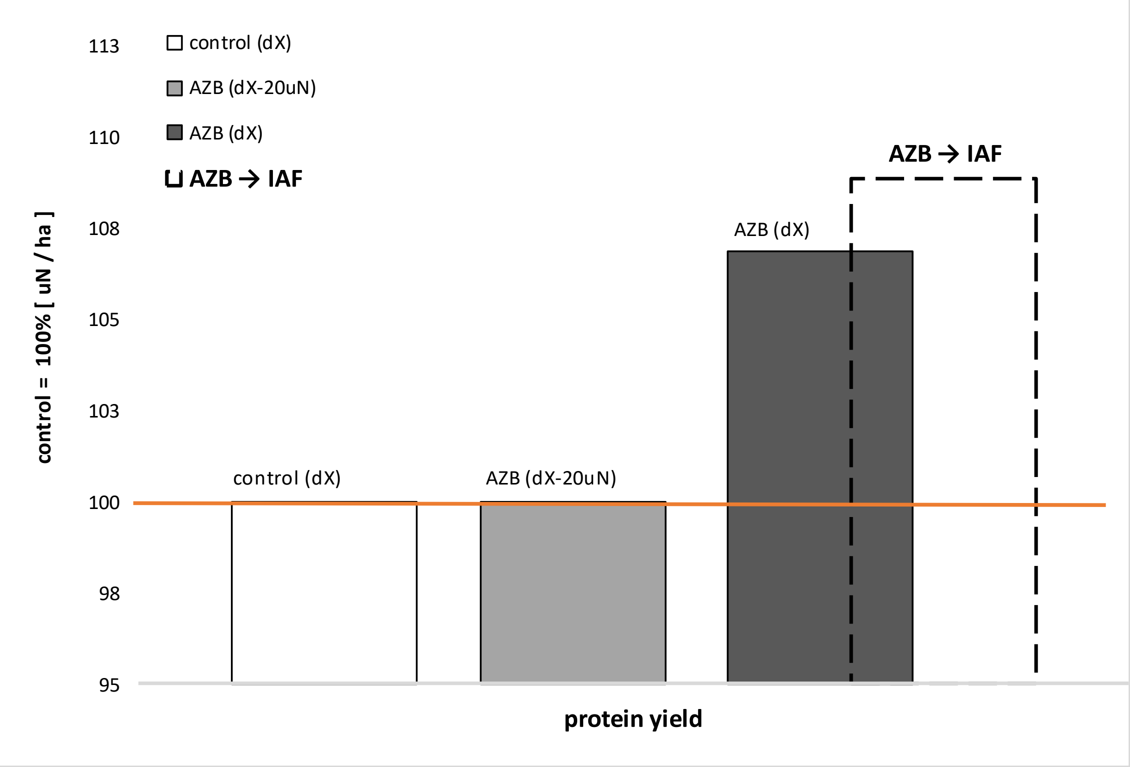

Figure 2 : The agronomic consequence of AZB → IAF (bis).

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[14] Data synthesized in Figures 1, 2 and 3 were obtained from field trails across France harvested from 60 site x years comprising close to 200 observed pair-wise comparisons.

AZB increases the use efficiency of N-fertilizers ($NUE_{I/O}$[15]) and will contribute to soil conservation by mitigating the priming effect of excess soil mineral nitrogen (Nm) on soil organic matter mineralization. This increased $NUE_{I/O}$ will also reduce this amount of post-harvest residual Nm and its contribution to NPSP[16]. Necessarily, IAF will also further increase the efficacy of AZB in terms of $NUE_{I/O}$ (**Figure 3**). The agronomic benefit in terms of grain-protein yield and $NUE_{I/O}$ is estimated at around +5 to +8% when compared to the non-AZB check. AZB integrating various existing nutritionals (AZB^+) should readily increase this benefit to ~12% and beyond.

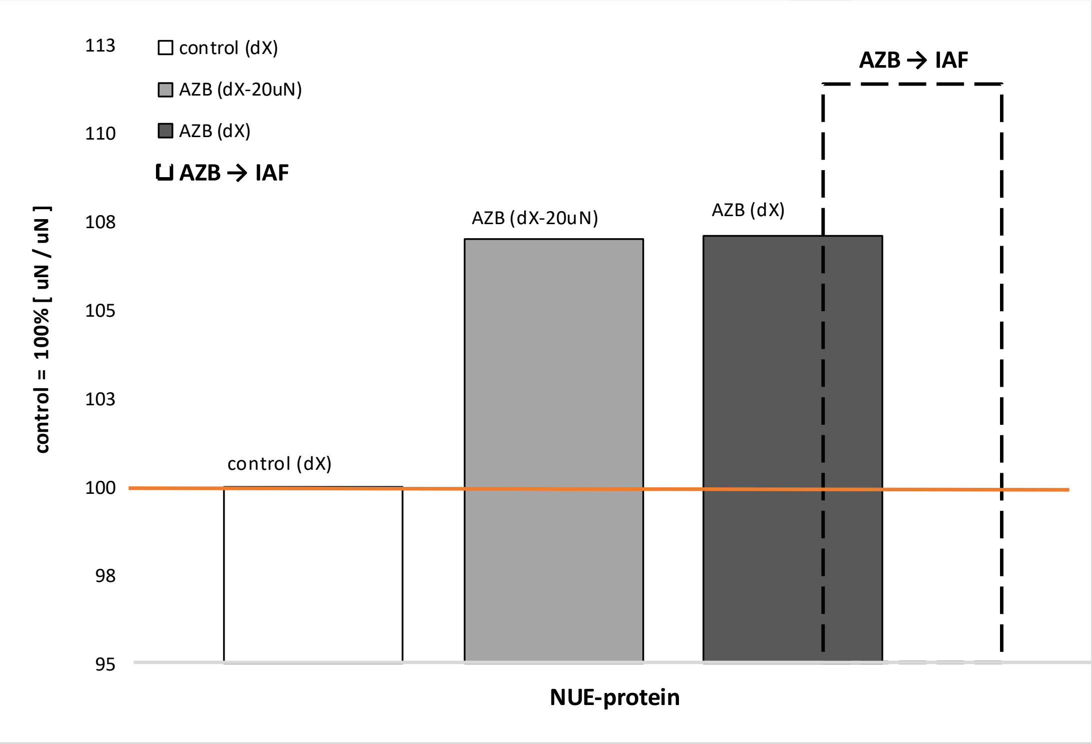

Figure 3 : The agronomic consequence of AZB and IAF (tertio).

The agronomic outcome of these trials is summarized in Figures 1, 2 and 3 herein. Briefly and as seen in **Figure 1**, integrated AZB → IAF using especially calibrated doses of fertilizer-N (dN) and a various N, P, K and S complementary nutritionals has been assayed in situ *and* in silico by Polyor since 2016. AZB will increase winter field crop (bread wheat in this case) despite a reduction (- 20 uN) in the amount of N-fertilizer applied (dX). This scenario is a priori the most intuitive given azotobacteria's ability to biologically "fix" dinitrogen. However, when the full dose (dX) of N-fertilizer is applied, the efficacy of AZB is even greater (a). We found this latter scenario to be agronomically more profitable given that grain protein content (%) was maintained (data not shown) allowing for a just as significant increase in protein yield (see below Figure 2). IAF will allow for a further increase in yield (***dIAF = b – a***) attributable to AZB.

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[15] Nitrogen fertilizer use efficiency expressed simply as the [Nf input / Protein-N output] ratio (I/O). A crude estimate of NUE, but nevertheless the most widely used in situ if only for practical reasons; cf. Fertilizers Europe. 2010. "Nitrogen use efficiency as an agro-environmental indicator" www.fertilizerseurope.com

[16] Non-point source pollution

Azotobacterial fertilization, AZB™, and yield stability

Field variability maps describing in-field yield variability attributable to the soil's spatial heterogeneity are now commonly proposed to farmers. This is usually referred to as precision agriculture applying "just what is needed, where it is needed" to minimize leakages to the environment. However, the agronomic consequences of these cards - technological jewels - are not always present in terms of yields. Their recovery also requires adapting fertilizer spreaders with sensors to precisely adjust their flow rates. This precision agriculture is more useful and profitable in crop protection, less in fertilization.

More simply, Polyor proposes *azotobacterial fertilization* (AZB™) capable of partially leveling the *post-harvest residual mineral-N* (PHN) variability. These azotobacteria will either over-immobilize large PHN and release them later-on in the season and/or biologically fix the atmospheric dinitrogen if and when PHN are low. This de facto modulation of the N available to the crop renders less imperative the use of variable N-fertilizer rates.

The in-field yield variability is largely due to the heterogeneity of *post-harvest residual-N* (PHN). In precision agriculture, this variability can be mapped – very indirectly via NDVI-like greening indices. N-fertilizer application rates are then modulated accordingly. However, the effectiveness of such variable rates, in principle providing just what is needed where it is needed, depends on sometimes tenuous satellite links, high-performance 5G sensors, suitable spreaders, etc.

Azotobacteria can indeed *sense* if there is little mineral nitrogen locally and fix even more dinitrogen. On the contrary, if the PHN are plentiful, they will immobilize it and release it later in the winter. According to Polyor, in the future it is the azotobacterial inoculation of straw crop residues as carbonaceous substrates for the non-symbiotic biological N-fixation of azotobacteria in azotobacterial fertilization – AZB™ - which will better ensure this uniformity.

This ability of such azotobacteria inoculated onto to soil borne crop residues to *over-immobilize* PHN as well as the subsequent increase in non-symbiotic N-fixation have been observed (EP2942621) leads to a certain level of yield uniformization and stability. For example, herein (**Figure**) winter wheat yields across the field at 10 or 30 meters intervals as attributable to various forms - AZB, Azb & azb - of azotobacterial fertilizations (AZB™) ;

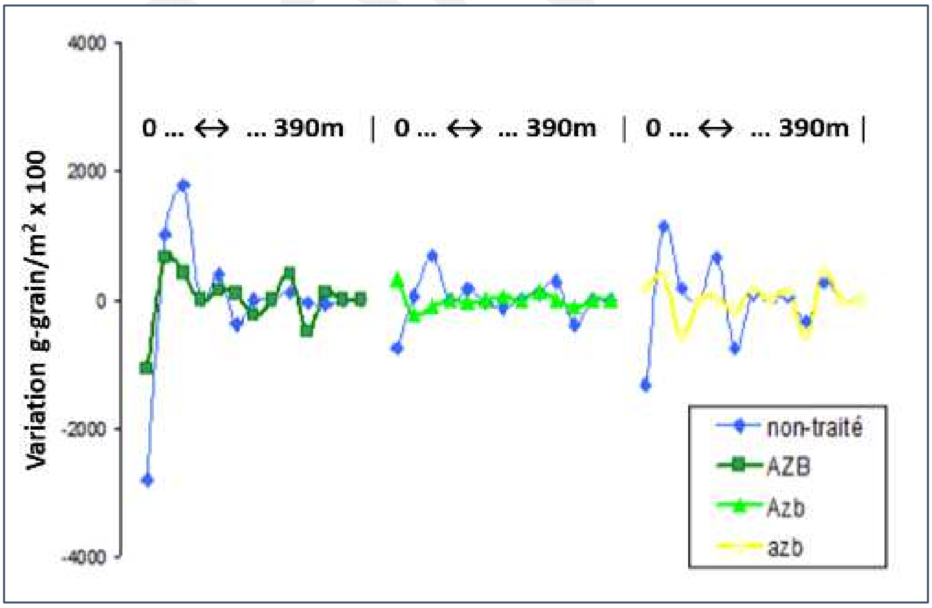

Figure. Variation in winter wheat yields as comparted to the overall mean on AZB™-treated plots™ with three different types of prototypic inocula (AZB, Azb & azb) and non-treated (*non-traité*) paired plots. Each point represents the variation in grain yield at 30m intervals expressed in g-grain/m² x 100.

ANNEX: TECHNICAL REPORTS OF 2011-2015 AGRONOMIC TRAILS.

AZB ON WINTER CEREALS (WHEAT): 2011, 2012 AND 2013 PAIRED-PLOT COMPARISONS DEMONSTRATING THE EFFICACY OF AZB: Field trails harvested in France in 2011, 2012 and 2013 to assess the efficacy of AZB on winter field crops such as wheat, were carried out. More than 200 paired-plot comparisons involving nearly 400 observations of grain (GYL) and protein (PYL) yield and NUE were analyzed; more than half of these comparisons included the reduction (rationing) of the recommended Nf dose (aka dX). AZB increased GYL and PYL by approximately 5 to 8%; NUE was increased by up to 10 percent on average. These increases were equivalent to a 60 to 70€ economic gross benefit. However, the rationing of dX was counterproductive because of a transient supra-immobilization of soil mineral nitrogen (Nm). Polyor has since proposed formulations and application strategies aimed at overcoming and exploiting this supra-immobilization of Nm.

AZB ON WINTER CEREALS (WHEAT): 2014 – FALL RESIDUAL MINERAL NITROGEN (rNm) : In theory, the azotobacteria on or near buried soil crop residues (SCR) favor this supra-immobilization of rNm. If this supra-immobilization could be amplified and lengthen, the efficacy of AZB could eventually be further improved. To this effect, a combination of dextrose, Mo, tryptophan and cations was applied directly to the SCR along with AZB immediately prior to their soil incorporation (burial). A simple but robust index of azotobacterial activity in the residusphere (detritusphere) - rNN, decreased in the fall three weeks after SCR burial, and then increase three months later. As predicted, this is concomitant with an initial increased immobilization of N-NH4 in the fall and proportional increased release of in the spring.

AZB ON WINTER CEREALS (WHEAT): 2014 - GRAIN, PROTEIN AND NITROGEN USE EFFICIENY (NUE): The positive effect AZB on yield and protein should have increased given the effect of dextrose combined with metal and amino acid cofactors on AZB's supra-immobilization of rNm. Instead, AZB efficacy was attenuated across all fertilizer treatments including the deferred Nf applications and foliar-N (Nfol). Since complementary Polyor technologies for isolating gluco-reactive azotobacteria in synergy with such dextrose additions were not used by the licensee the neighboring non-necessarily nitrogen fixing bacteria were triggered at the expense of the inoculated AZB azotobacteria. This resulted in supra-immobilization as predicted, but with no immediate benefit in terms of increased AZB efficacy. Interestingly, this demonstrated not only the efficacy of AZB alone, and dextrose's potency at triggering soil bacteria, but also the complementarity of Polyor's portfolio.

AZB ON WINTER CEREALS (WHEAT): 2015 – FALL RESIDUAL MINERAL NITROGEN (Nm): Despite the ambivalent effect of dextrose applied to soil borne crop residues (SCR) on AZB efficacy, the effect AZB's supra-immobilization of rNm on yield and NUE were looked at. The most pertinent way of demonstrating this effect of diverting supra-immobilized rNm to the latter stages of crop growth is using an Ancova with rNm as a quantitative covariable in addition to blocks (rank), columns and treatments as qualitative variables. rNm's partial regression coefficients for grain and protein yield were as predicted positive and significant only with AZB (+/- dextrose). rNm contribute positively to yield only if the SCR are AZB-treated. This contribution of rNm when SCRs are AZB-treated will supplement the N supplied to the soil-plant systems by non-symbiotic N-fixation. This analysis corroborates a previous 2011 observations concerning the per unit rNm yield of grain and protein.

AZB ON WINTER CEREALS (WHEAT): 2015 - GRAIN, PROTEIN AND NITROGEN USE EFFICIENCY (NUE): Further evidence of AZB efficacy - and dextrose's counter-efficacy, on yield and NUE is provided. Another foliar-N concept (Nfol) was assayed along with an exogenous azotobacteria as a negative control. Once again, dextrose likely triggered the activity the neighboring gluco-reactive bacteria seemingly at the expense of AZB's SCR-applied azotobacteria. Though various Nf fertilization treatments were assay as before in

2014 so as to render less arbitrary the sought-after reduction of dX, the efficacy of AZB is still the most easily detectable when dX is not tampered with needlessly. Having said this, there is most likely a very strong interaction between AZB efficacy and Nf dosage which needs to be taken into account when recommending dX for AZB-treated plots, i.e. the very purpose of Polyor’s IAF approach to sustainable agriculture involving the aggregation of indices of AZB efficacy by multicriteria analysis.

CLAUDE ET AL. COMMUNICATIONS REGARDING AZB

Claude 1997 (Ensat): The effectiveness of biofertilization by exploiting crop residues is currently limited by the non-persistence of microbial strains reintroduced into soils. In order to lift this limiting factor, culture media, in particular silicas gels, have been modified so as to allow the isolation of endogenous, competitive and more telluric microbial strains than those of more conventional azotobacterial inoculants. These novel inoculants prepared from microbial biomasses isolated on silica gels impregnated with humic and para-humic substances increase nitrogenase activity and mineralizable N content in the soil resulting in increases of 8 to 12 mg of mineralized N per kg of soil, or about 20 to 27 kg per hectare. This could represent in fine the equivalent of more than 20 percent of the $N_{fertilizer}$ requirements of winter wheat.

WO03046156 (A2) — 2003-06-05: A method for obtaining in vitro soil bacterial biomasses. This involves the contacting an aqueous soil suspension with a matrix substrate, so as to form a hydrated zone, or biofilm, at the surface of the substrate, similar to the one adjacent to the clay-humus complex of the soil, essentially containing the internal bacterial constituent of the soil, so as to retain at the surface of the matrix substrate, the essential part of the bacterial flora endogenous to the original edaphic media; maturing the biofilm providing oligotrophic conditions enabling the cells which constitute most of the edaphic biofilm which have colonized the matrix substrate to migrate to the supernatant liquid phase of the matrix substrate, and gradually to prevail over it; recovering and culturing the bacterial strains most prolific in rich liquid culture media, with internal character, rustic and persistent in situ, capable of being cultured in liquid environment, hence capable of industrial production. The resulting bacterial biomasses and strains are useful for microbiological bacterization of soils and crop residues, in particular cereal crop residues, including those of corn.

Claude et Fillion 2004: The object of this work is to further develop the use of PGPR (Plant growth promoting rhizo-bacteria) for non-*Fabaceae* crops such as spring and winter cereals. To this effect, a solid-state, non-wettable, inoculum containing novel PGPR strains isolated from French soils using an innovative and patented isolation protocol was developed. The in situ bacterial treatment of cellulosic crop residues, including those of cereals and grain corn, by spraying a suspension of the aforesaid inoculum should allow these residues to act as substrates for the re-introduced PGPR strains thus increasing the yield of winter wheat. Field trials in France over the 2002-2003 cropping season demonstrated that, given a well-managed intensive in-season nitrogen-fertilizer optimization protocol, it is effectively possible to increase the yield of winter wheats sown into inoculated grain-corn residues by approximately 200 to 300 kg per hectare, without any decline in protein or 1000 seed weight.

Claude et Giroux 2006: The use of banded starter fertilizers for corn is generally recommended even in relatively high soil P. However, the use of starter fertilizers further increases soil P thus reducing the possibility for environmentally secure manure applications. Increase P efficiency of starter fertilizers is thus a useful for the reduction P-fertilizer application. The agronomic use of organo - mineral fertilizers (OMF) with growth promoting bacteria (PGPB) and at seeding as starter fertilizers was tested for use in grain - corn on two soil type (podzol and gleysol). OMF-PGPB increased seedling development and dry-matter accumulation 34 days after seeding just as much as MAP containing two to three times more available P. However, OMF-PGPB is more effective on the gleysol, somewhat richer in Ca and Mg phosphates, than on podzol, rich in alumino-ferric P. In fact, grain yield increases attributable to OMF-PGPB were detected on the gleysol only.

FAR (Comifer) 2017 – Nantes: FAR (*alias* integrated azotobacterial fertilization, or IAF) is an alternative to the balance sheet method for calculating $N_{fertilizer}$ rates for field crops such as wheat. Indices – patented for the most part, of the *efficacy* of azotobacterial fertilization (eAZB) with soil borne crop residues are derived from existing pedoclimatic data and integrated by multi-criteria assessment into a single aggregated indicator of eAZB, iAZB™ (EP17196251.7). Taking into account target yield (pRDT), $N_{fertilizer}$ as dN calculated according to the equation dN = ax pRDT - pAZB decreases as a function of eAZB as a result of a an increase in NUE (a) and the N-contribution of the inoculated azotobacteria (pAZB). This method of calculating dN was applied to 30 existing datasets to evaluate dN in terms of relevancy and accuracy. It turns out that dN as derived from the IAF approach is in fact more determinate and precise than conventional $N_{fertilizer}$ rates.

FAR (Comifer) 2018 – gSAB : Since 2000 a series of azotobacterial fertilization (AZB) field trials were harvested in France. These involved the application of endogenous azotobacteria directly to soil-borne crop residues prior to the seeding of winter field crops such as wheat. We found that the systematic reduction of the $N_{fertilizer}$ recommended rates as sought after by the user is counterproductive and reduces AZB efficacy. In response, Polyor has since proposed to develop a sort of *integrated azotobacterial fertilization* (IAF) integrating (aggregating) a series of indices of AZB efficacy. Using IAF we can recalculate dX considering this potential efficacy of AZB. However, some of these indices affected by soil pH and pertaining to bacterial biogeography (iAPC) and/or the resiliency of soil bacteria confronted with pH variations (iSAB) need to be better assessed. It turns out that, unlike azotobacteria abundance and diversity in themselves, the effectiveness of AZB is not simply a function of pH ; i.e. in terms of AZB efficacy, there is no good or bad soil pH but rather a certain degree of adaptation and endogeneity of azotobacteria as indicated by iAPC and iSAB. These two indices of AZB efficacy are then aggregated into a single composite index, iAZB, used to predict AZB efficacy and recalculate $N_{fertilizer}$ (dN) more precisely by considering the contribution of AZB.

FAR (Comifer) 2018 – gPKMg : Azotobacterial fertilization (AZB) was initially developed in the USSR at the beginning of the 20th century, but was shelved in the West in the late 1930s. Polyor is reintroducing AZB in France and in Europe. However, these azotobacteria have high energy requirements and consume a lot of ATP and P. The problem is that free living azotobacteria only exploit water-soluble P, not the so-called exchangeable P (eg P-Olsen, etc.). In addition, a there is a correlation between water soluble P and the accumulation of N derived from non-symbiotic N-fixation. Although azotobacteria cannot extract soil P, they will benefit from P applied directly to soil borne crop residues because. Polyor modelled of the potential increase in azotobacterial activity as a function of this P enrichment of the *residusphere*. These microdoses P (≈ 1 kg-P/ha) applied directly to the aforesaid crop residues supply P to the azotobacteria. This can be done with conventional liquid foliar P-fertilizers sprayed directly onto the straw before burial. This is a new opportunity for liquid fertilizer manufacturers and a new avenue for existing foliar applied N and P nutritionals. The effect of these microdoses of P on AZB efficacy can be considered when recalculating the $N_{fertilizer}$ rates according to the aforesaid AZB → IAF approach.

FAR (Comifer) 2018 – gNS : The balance sheet method presents some problems of precision, redundancy and bias. Indeed, the non-symbiotic fixation is neglected in the balance sheet method whereas studies show that it may account for some 25 kg of N per hectare per year. Trials were conducted from 2011 to 2015 on 60 farm plots with more than 200 matched comparisons or azotobacteria fertilizations as azotobacterial fertilization (AZB) were made on post-harvest straw residues. The in situ efficacy of AZB has been proven; it allows gains in protein yields and an improvement in NUE. However, this should not necessarily lead to a decrease in N-fertilizer application rates but rather to improve N and AZB efficiency and even to surpass average crop yield. Polyor works to improve AZB as part of a so-called integrated azotobacterial fertilization (IAF) to fine tune N-fertilizer application rates by taking into account the potential of AZB.