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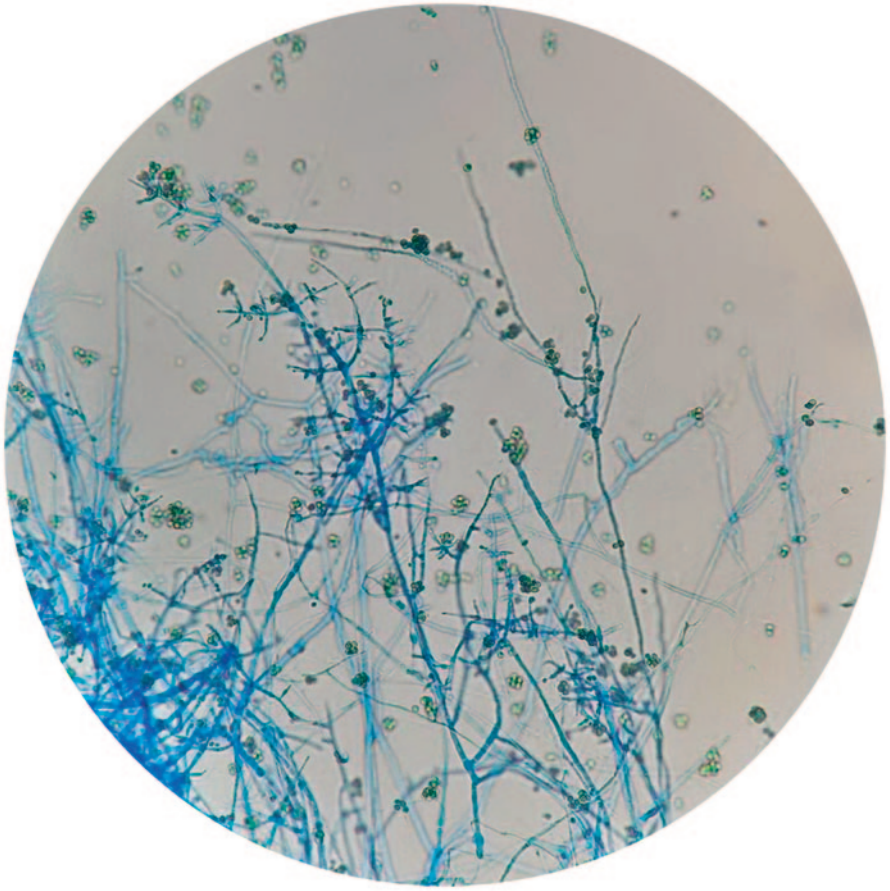
facts

CITROFOL® AI as a carrier fluid for microbials



Introduction

Microbials in agriculture

Biocontrol agents and biostimulants improve the resilience of plants against biotic and abiotic stressors, and are thus important sustainable crop inputs in agriculture. In particular, microbial agents offer a variety of beneficial interactions with plants. Commercially significant microbials include the Gram-positive rhizobacterium *Bacillus velezensis*, which produces antimicrobial compounds that are active against plant pathogens and can trigger induced systemic resistance, thus providing both biocontrol and biostimulant action.[1] The same applies to the fungus *Trichoderma harzianum*, which mobilises nutrients and protects against soil-borne diseases such as *Rhizoctonia* spp., *Fusarium* spp. and *Sclerotinia* spp.[2] The entomopathogenic fungus *Beauveria bassiana* is a widely used fungal biocontrol agent with efficacy against pests such as whitefly, the European corn borer and the Colorado potato beetle.[3]

However, maintaining cell viability is a persistent challenge, even for currently marketed microbial products. In many cases, products have short shelf lives and require cooling, and as a consequence, application may be inefficient. Formulation is a key factor for tackling this challenge. Many products come as wettable powders, but liquids are often preferred as they are dust-free, easier to dose, rapidly dispersible in water and compatible with filters. Carrier fluids for microbials need to provide a low-water activity environment to ensure cell viability. Because of this, aqueous carriers are often unsuitable and non-aqueous fluids are preferred.

The goal of the present study was to evaluate the potential of CITROFOL® AI to function as a carrier fluid for three commercially significant microbials (*Bacillus velezensis*, *Trichoderma harzianum* and *Beauveria bassiana*), focusing on its impact on cell viability. We also aimed to develop a protocol for adjusting the rheology of CITROFOL® AI to ensure stable suspensions. Finally, a greenhouse study was conducted to confirm that CITROFOL® AI is compatible with plants.

Benefits of CITROFOL® AI as a carrier fluid for microbials

CITROFOL® is Jungbunzlauer's globally recognised brand of citrate esters. The CITROFOL® range comprises clear, colourless and odourless oily liquids. One of these citrate esters is triethyl citrate, sold as CITROFOL® AI, which is produced by esterification of raw materials derived from fermentation and is thus 100% bio-based and biodegradable.

The physicochemical properties of CITROFOL® AI give it distinct benefits as regards handling and formulation (table 1). Firstly, it is non-flammable, non-volatile* and non-toxic, and therefore safe to store and handle. It offers water solubility of up to 58 g/L, allowing it to homogeneously dilute into water at relevant use rates, thus obviating the need for emulsifiers and allowing simpler formulations. It is stable at cold temperatures and well pourable over a wide temperature range, with a viscosity of 27 mPa·s at 25°C and 400 mPa·s at –10°C across shear rates.

*As defined by Directive 2004/42/EC

Table 1: Overview of relevant physicochemical characteristics of CITROFOL® AI

Molecular weight [g/mol]	276
Density [g/ml]	1.14
Boiling point [°C]	287
Flash point [°C]	178
Viscosity [mPa·s at 25°C, 100 s^{-1}]	27
Vapour pressure [mbar at 25°C]	0.0025
Interfacial tension [mN/m]	32
Wetting/spreading	Medium
Hansen solubility (δ_D / δ_P / δ_H)	16.5 / 4.9 / 12.0
Water solubility (g/L)	58.1
Hydrolytic resistance (DIN 53402)	610
Odour	Odourless
Appearance	Clear, colourless liquid

CITROFOL® AI is listed in international chemical inventories and is registered under the EU REACH Regulation. In the USA, triethyl citrate is approved as an inert ingredient for use in pesticide products applied to food and non-food, respecting the limitations set by 40 CFR Part 180. CITROFOL® AI is also USDA Certified Biobased under the USDA BioPreferred® Program, and is suitable for use in bio-based applications in designated categories.

Materials and methods

Viability tests

To assess the impact of CITROFOL® AI on microbial viability, commercially significant microbials were obtained from the market as formulated products. These comprised the Gram-positive bacterium *Bacillus velezensis* (wettable powder and aqueous suspension concentrate) and the fungi *Trichoderma harzianum* and *Beauveria bassiana* (both formulated as wettable powder). The powder products were suspended in distilled water amended with 0.2 wt% Tween® 80, and the samples were thoroughly vortexed to ensure cell dispersion. A tenfold serial dilution was performed, with the suspensions being streaked onto Luria-Bertani agar plates (*B. velezensis*) or potato dextrose agar plates (*T. harzianum* and *B. bassiana*) in duplicate. Plates were incubated at room temperature and colony-forming units (CFUs) were counted to determine the products' starting viability.

To test storage stability, cell suspensions were prepared in CITROFOL® AI by suspending the wettable powder products at a use rate of 10 wt%. All of the formulations and commercial products were stored at 40°C and/or room temperature for up to six months, and cell viability was periodically measured by plate counting as described previously.

Rheology modification

To facilitate development of formulations, rheology modifiers were screened regarding their suitability for thickening CITROFOL® AI. This was achieved by dispersing hydrophobic fumed silicas in CITROFOL® AI at a use rate of 3.0 or 4.5 wt% by homogenisation for ten minutes. The flow curve was measured by rotational viscometry (Haake Rheostress, plate-cone geometry C35/2° Ti L). Because of the samples' shear thinning behaviour, they were allowed to rest for a period of 10 min after application onto the rheometer platform before measurement was started. The apparent viscosity was determined at continuously increasing shear rates from 0.01 to 1000 s^{-1}.

Greenhouse study

To prove that CITROFOL® AI is compatible with plants and does not interfere with the efficacy of microorganisms, a greenhouse study was conducted using maize (two plants per pot, six replicate pots per treatment, three plots in a randomised design). Seeds were placed into pots containing a 1:1 blend of sand and soil collected from non-fertilised grassland, with a pH of 8.3 and a low level of plant-available P. The substrate was supplemented with non-plant-available P (calcium phosphate) to 25–35 ppm. The N and K content was adjusted to 80 mg/kg and 150 mg/kg respectively (setup based on Eltlbany *et al*.[4]). *T. harzianum*, which is known to mobilise P and thus promote plant growth under low-P conditions, was applied to the maize upon sowing and after germination by diluting a test formulation of *T. harzianum* in CITROFOL® AI or a reference wettable powder product in water according to the manufacturer's instructions and applying these formulations at a rate of 1.5 x 10^7 CFU/plant. Plants were cultivated in a growth chamber (17–24°C, 16 h light/8 h darkness) for two weeks, then transferred to an open greenhouse. The plants were irrigated from the bottom to prevent the applied microbials from draining away. Germination rate and agronomic parameters (plant height, above-ground biomass, root length and biomass) were measured four and eight weeks after germination. A statistical analysis was performed using the SPSS 28 software package (ANOVA with post-hoc Tukey test).

Results

Validation of cell viability method

The starting viabilities of the original microbial products, determined as colony-forming units (CFUs), were in line with the respective manufacturers' specifications. This was an important underlying observation for the shelf-life study, confirming the suitability of the protocol used for microbial cultivation and plate counting. Additionally, the cell counts in the original products were the same as in the CITROFOL® AI formulations when measured directly after blending. This underlined the good dispersibility of the cells in CITROFOL® AI even in the absence of a dispersing agent, resulting in a high validity of cell counts. The partial water solubility of CITROFOL® AI simplifies the procedure, since the cell suspension can be directly transferred to an aqueous serial dilution. This is a benefit compared to viability measurements in common oily carriers, which require the addition of surfactants and centrifugation to transfer cells from oil to an aqueous medium.[5]

Cell viability of *B. velezensis*

The viability of the spore-forming bacterium *B. velezensis* was monitored at a challenging storage temperature of 40°C. Despite these harsh conditions, the *B. velezensis* wettable powder product proved robust and fully maintained its viability throughout the six-month test period. However, this was not the case with the commercial *B. velezensis* liquid product – an aqueous suspension concentrate – in which cell viability dropped from 1.5 x 10^{10} CFU/g to 4.3 x 10^{8} CFU/g over six months at 40°C. Although not as robust as the wettable powder formulation, suspension in CITROFOL® AI did deliver an improved shelf life for liquid product formats: The viability of *B. velezensis* declined slightly from 1.5 x 10^{9} CFU/g to 4.8 x 10^{8} CFU/g, less than one order of magnitude, during six months at high temperatures (figure 1).

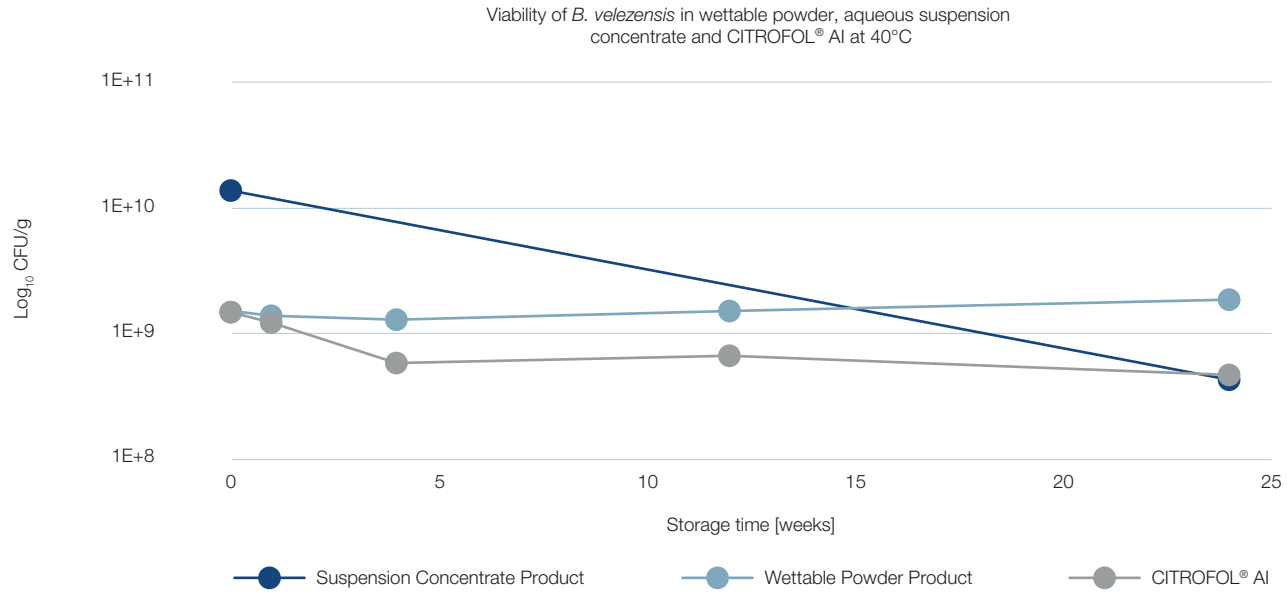


Figure 1: Cell viability of *B. velezensis* stored at elevated temperatures for an accelerated storage test, comparing a commercial wettable powder with a commercial aqueous suspension concentrate and a non-aqueous suspension in CITROFOL® AI. Differences in starting viability are due to different cell concentrations in the products.

Cell viability of *T. harzianum*

T. harzianum proved more susceptible to high storage temperatures, but shelf-life monitoring at 40°C for six months was nonetheless feasible. There was a pronounced drop in cell viability in the wettable powder product over this period, from 1.8 x 10^9 CFU/g to 5.1 x 10^4 CFU/g. Suspension in CITROFOL® AI attenuated this loss: the CITROFOL® AI suspension had a viability of 2.7 x 10^6 CFU/g at the end of the measurement period, almost two orders of magnitude higher than the reference powder product.

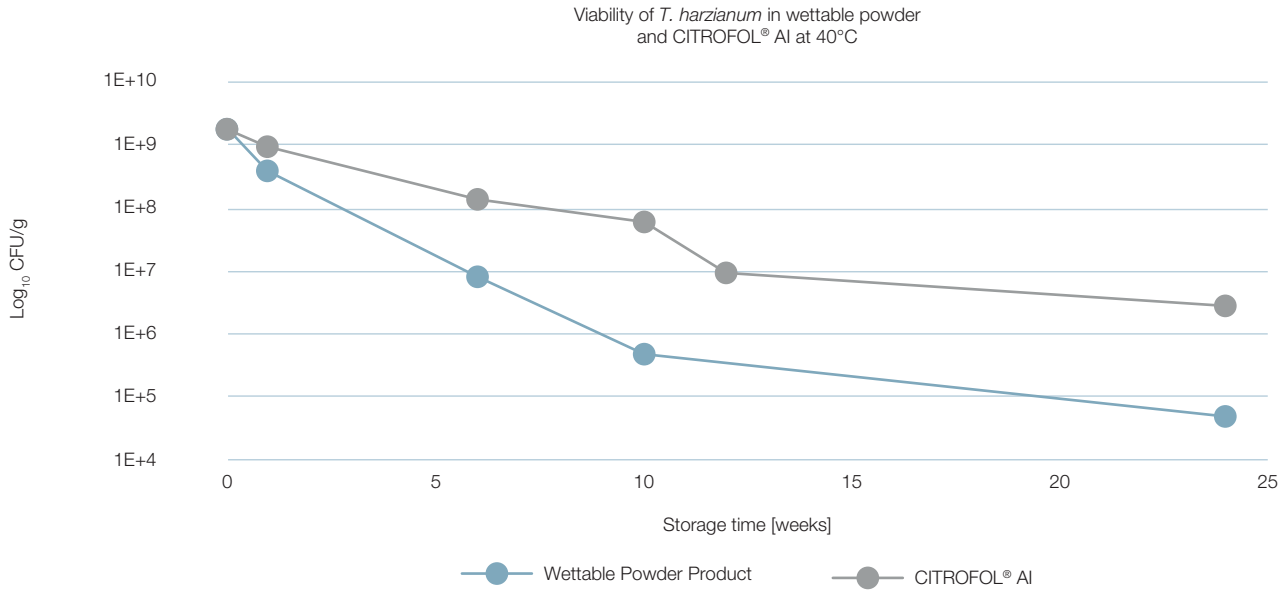


Figure 2: Cell viability of *T. harzianum* stored at elevated temperatures for an accelerated storage test, comparing a commercial wettable powder product with a suspension in CITROFOL® AI. Data point at 12 weeks for the wettable powder was below the detection limit.

Cell viability of *B. bassiana*

Among the microorganisms tested in the present study, *B. bassiana* was the most sensitive to elevated storage temperatures. To provide better resolution, shelf life was monitored both at room temperature and 40°C. After just two weeks at 40°C, cell viability in the wettable powder product had dropped from 5.3 x 10^8 CFU/g to below the detection threshold of approx. 1.0 x 10^3 CFU/g. Under the same conditions, the suspension in CITROFOL® AI had only decreased slightly to 1.1 x 10^8 CFU/g after two weeks, and was still as high as 1.0 x 10^6 CFU/g after twelve weeks (figure 3). As expected, viability declined more slowly when *B. bassiana* formulations were stored at room temperature. Under these conditions, cell counts in the wettable powder product dropped from 5.3 x 10^8 CFU/g to 4.1 x 10^7 CFU/g after 24 weeks. Shelf life was better still in CITROFOL® AI, where cell counts were 2.5 x 10^8 CFU/g at the start and 1.6 x 10^8 CFU/g at the end of the test period (figure 4). Our observations are in line with published results showing an increased tolerance of *B. bassiana* to elevated temperatures when formulated in an oily carrier.[6]

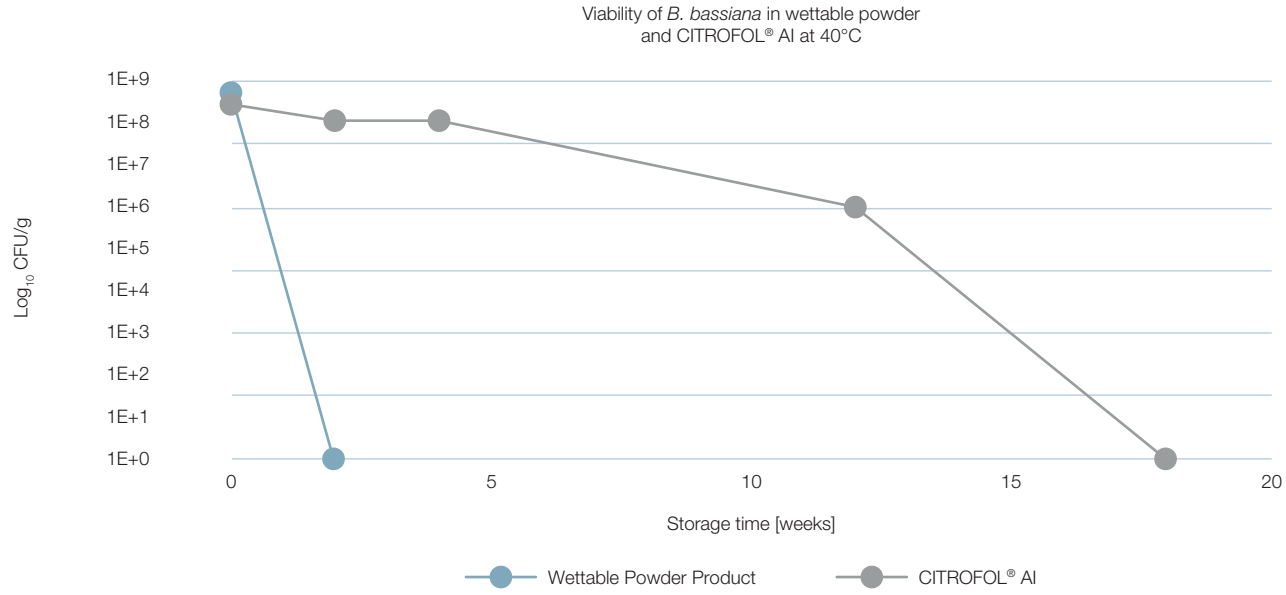


Figure 3: Cell viability of *B. bassiana* stored at elevated temperatures for an accelerated storage test, comparing a commercial wettable powder product with a suspension in CITROFOL® AI

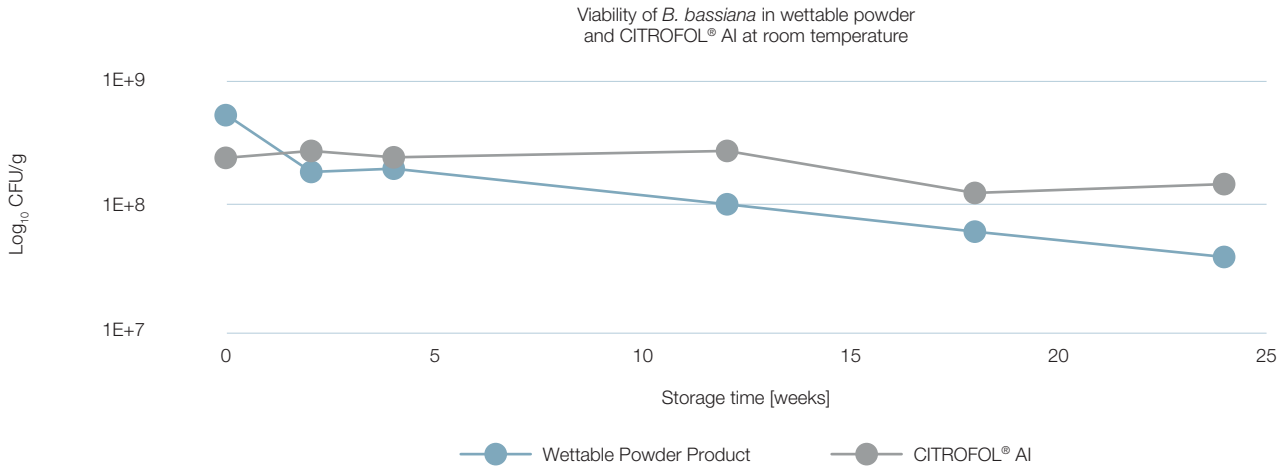


Figure 4: Cell viability of *B. bassiana* stored at room temperature, comparing a commercial wettable powder product with a suspension in CITROFOL® AI

Optimising rheology

The formulation-relevant physical characteristics of a carrier fluid are an important consideration. Adding hydrophobic fumed silica at a low use rate of 3.0 wt% was sufficient to modify the rheological characteristics of CITROFOL® AI in the desired way. The apparent viscosity of the thickened fluid was around 100,000 mPa·s at a low shear rate of 0.01 s^{-1}, indicating that dispersed cells could be very well stabilised against sedimentation. At the same time, the thickened fluid displayed strong shear thinning properties, which was apparent when the shear rate was increased to 10 s^{-1}, resulting in a 100-fold reduction of viscosity to around 1,000 mPa·s (figure 5). These values show that formulations based on thickened CITROFOL® AI have good pourability and dosability.

The stabilisation capacity of the formulation was tested using talcum powder as a model particulate substance. The samples were stored at 40°C for seven months. After this time, only slight separation had occurred at the top of the sample thickened with 3.0 wt% hydrophobic fumed silica. Stirring or shaking was sufficient to reverse this slight phase separation. The sample thickened with 4.5 wt% hydrophobic fumed silica was stable at 40°C for over seven months (figure 5).

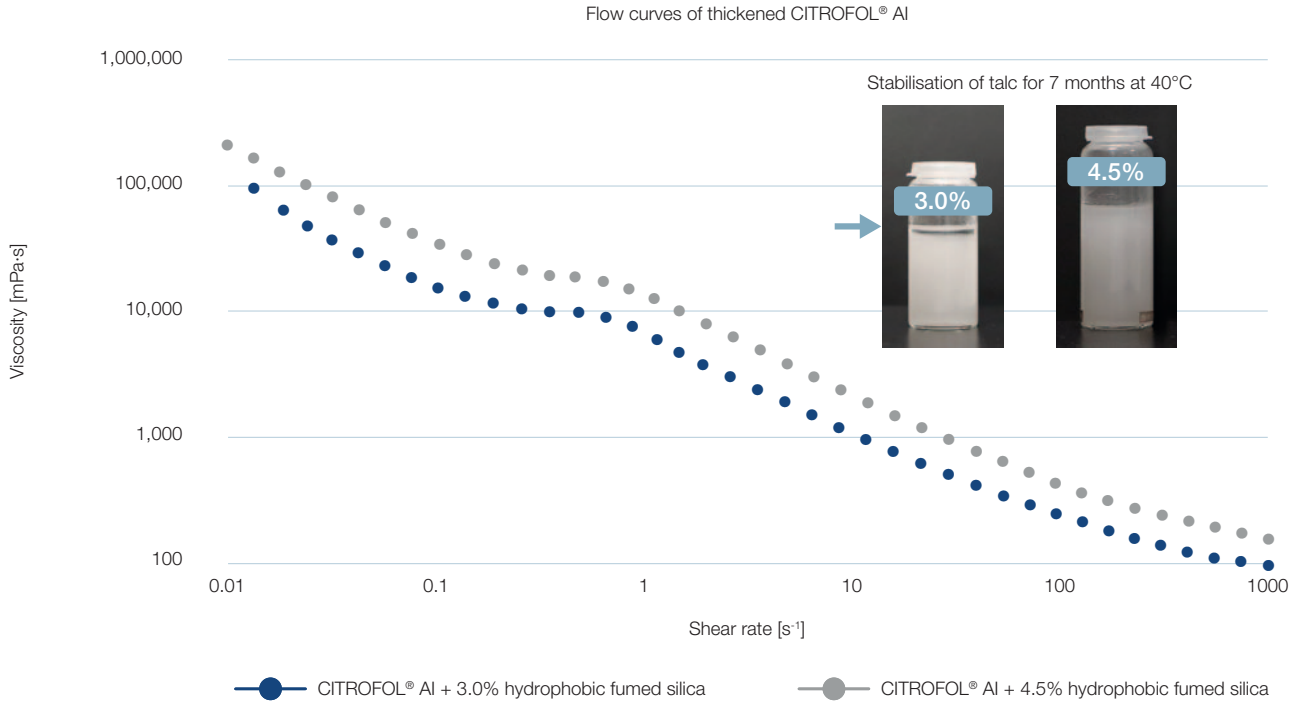


Figure 5: Flow curves of CITROFOL® AI thickened with different amounts of hydrophobic fumed silicas. The images show the condition of the suspensions after being stored at 40°C for seven months, with talcum powder used as a model particulate substance.

Greenhouse study

Finally, it is important that co-formulants are plant-compatible and do not interfere with the efficacy of the microbial application in question. This was assessed by comparing the development of maize plants treated with water (negative control), *T. harzianum* wettable powder product (positive control) and *T. harzianum* dispersed in CITROFOL® AI. No significant difference was found in any of the agronomic performance parameters tested (germination, root and shoot growth). Since the positive control did not exhibit the expected beneficial effect on plant development, the results do not allow for drawing definite conclusions regarding the non-interference of CITROFOL® AI with microbial efficacy. However, since the agronomic parameters did not differ significantly between the negative control and the treatment with CITROFOL® AI either, it can be concluded that CITROFOL® AI is a plant-compatible carrier fluid.

Conclusion

Our results prove the suitability of the citrate ester CITROFOL® AI in the novel function as a carrier fluid for microbials. CITROFOL® AI is a sustainable co-formulant, is very safe and boasts favourable physicochemical characteristics. In particular, it combines the low water activity of an oily carrier with partial water solubility, thus making emulsifiers and preservatives obsolete and ultimately simplifying formulations. CITROFOL® AI is fully plant-compatible and has significant potential to improve the shelf life of liquid microbial formulations, as shown with all organisms tested in this study (*B. velezensis*, *T. harzianum*, *B. bassiana*). It helps to maintain high cell viability even at elevated temperatures, which is important for farmers working in warm climates or where continuous cooling of the product during transport and storage is not feasible. Ensuring high cell viability and long shelf life is a major step in overcoming constraints in the commercialisation of microbials. Viable microbial cells are indispensable for obtaining satisfying results in the field and thus boosting confidence in the benefits and economic feasibility of these sustainable crop inputs.

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Jungbunzlauer is one of the world's leading producers of biodegradable ingredients of natural origin. We enable our customers to manufacture healthier, safer, tastier and more sustainable products. Thanks to continuous investments, state-of-the-art manufacturing processes and comprehensive quality management, we are able to provide outstanding product quality.

Our mission "From nature to ingredients®" commits us to the protection of people and their environment.

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