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15 December 2023

Bernadette Juarez
U.S. Department of Agriculture
APHIS Deputy Administrator
Biotechnology Regulatory Services

Contains Confidential Business Information– 23-228-02

Dear Ms. Juarez,

Nuseed Nutritional US Inc, (herein referred to as "Nuseed"), a wholly owned subsidiary of Nufarm Limited, is submitting a revised request for Regulatory Status Review of a genetically engineered *Brassica juncea* for production of docosapentaenoic acid (DPA) and other long chain omega-3 fatty acids under USDA APHIS 7 CFR part 340 as per the request from Mr. Tangredi on 7 Dec 2023.

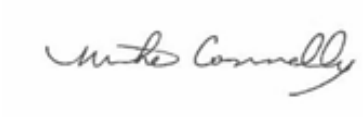
The subject of this request is to update the Confidential Business Information (CBI) captioning and justification pertaining to the review of the construct for production of DPA in *B. juncea*. These updates are not expected to impact the ongoing scientific review of this request.

CBI Justification

Information marked by Nuseed as CBI is exempt from release and/or public disclosure under Freedom of Information Act (FOIA) Exemption 4, 5 U.S.C. Section 552(b)(4), which states that commercial and financial information that is both customarily and actually treated as private by its owner and is provided to the government under an assurance of privacy, thereby marked as "CBI". Release of information marked as CBI would provide competitive information about the nature of the research, development, and commercialization plans of Nuseed and could jeopardize protection of its intellectual property rights. Access to this information would cause competitive and/or financial harm to Nuseed.

If you have any questions pertaining to this request, feel free to contact me.

Best regards,



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Information Supporting a Regulatory Status Review of Genetically Engineered *Brassica juncea* for production of DPA and other Long Chain Omega-3 Fatty Acids

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Introduction

Nuseed Nutritional US Inc. (herein referred to as "Nuseed"), a wholly owned subsidiary of Nufarm Limited, is submitting information to USDA APHIS to support a Regulatory Status Review for *Brassica juncea* that has been genetically engineered to produce oil containing omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LCPUFAs), with a significant amount of docosapentaenoic acid (DPA, 22:5 ω3).

The ω3 LCPUFAs eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are nutrients widely recognised for their roles as essential components of cell membranes and of particular importance for cardiovascular, cognitive and inflammatory health (Simpolous, 2006). The third most prevalent ω3 LCPUFA in fish is docosapentaenoic acid (DPA, 22:5 ω3). Present at 1-3% of fatty acids in natural fish oils, its abundance falls well below EPA and DHA which comprise 20-30% of total fatty acid in fish oil.

Recently, there has been increasing interest in the intermediary fatty acid, DPA, hypothesized not only to serve as a reservoir for EPA and DHA but also as an important precursor to a family of molecules known as specialized pro-resolving mediators, or SPMs. These compounds are involved in the resolution of inflammation and in regulation of immune function (Vik et al., 2017; Drouin et al., 2019; Fard et al., 2021). As such they may play roles in inflammation-related gut, cardiovascular, joint, skin and neural conditions and may act in concert with other ω3 LCPUFAs or play distinct roles.

The development of a plant based DPA oil could make an important contribution to the supply of DPA for further evaluation of its health benefits and create a sustainable source of this ω3 LCPUFA for human and animal nutrition.

The subject of this submission is for Regulatory Status Review of the genetically engineered event, NUBJ1207 and for the DPA construct pJP3662, under 7 CFR Section 340. The introduced genes are identical to those already present in the DHA canola event NS-B50027-4, which expresses an additional delta-4-desaturase to convert DPA to DHA (**Figure 1**). APHIS has already reviewed and concluded the DHA canola event and progeny derived from it are unlikely to pose a plant pest risk and therefore are no longer subject to the Regulations under 7 CFR part 340. USDA also concluded that DHA canola will not be a significant impact, individually or cumulatively, on the quality of the human environment (17-236-01p). Accordingly, APHIS concluded nonregulated status for DHA canola.

The DPA expression pathway can be considered a subset of the deregulated DHA canola as it expresses the same pathway but with one fewer fatty acid enzyme, ending at the production of DPA vs DHA (**Figure 1**).

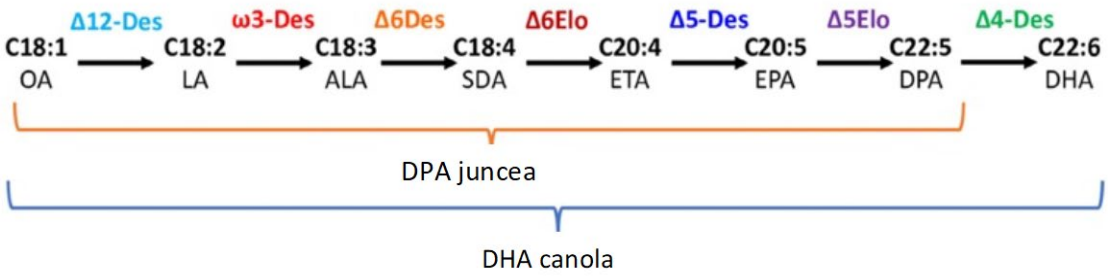

Figure 1. Biosynthesis pathway of NUBJ1207 and pJ3662 (DPA juncea) versus NS-B5ØØ27-4 (DHA canola).

For your review, a summary of information on the comparator plant, genotype of DPA juncea, with emphasis on the long chain omega-3 fatty acid pathway genes and their functions, the T-DNA sequence

of both NUBJ1207 and the pJP3662 construct, the intended trait and phenotype, and the mechanism of action (MOA) of the trait are provided in this submission.

Useful Definitions

NS-B50027-4 DHA canola event, produced via transformation of pJP3416_GA7-ModB construct, contains full set of 8 expression cassettes, produces DHA and other omega-3 fatty acids in seed oil, previously granted nonregulated status by APHIS.
NUBJ1207 DPA juncea event, produced via transformation of pJP3416_GA7-ModB construct, **[ ]**, produces DPA omega-3 fatty acids in seed oil. **CBI-del**
pJP3416_GA7-ModB construct contains 8 expression cassettes, used for transformation of DHA canola and DPA *juncea*.
[ ], to be used to transform *B. juncea* for DPA production. **CBI-del**

1 Description of *Brassica juncea*, the comparator plant

Scientific name: *Brassica juncea subsp. juncea*
Family: *Brassicacea*
Genus: *Brassica*
Species: *juncea* (2N=36)
Subspecies: *juncea*
Common name: mustard, brown mustard, oilseed mustard, canola

The origin of *B. juncea* is believed to be interspecific crosses between plants of *B. rapa* and *B. nigra* about 10,000 years ago (**Figure 2**). One primary center of origin is thought to be China with a second center in Afghanistan, the latter being the source of spread to the Indian subcontinent where it became a major oilseed crop.

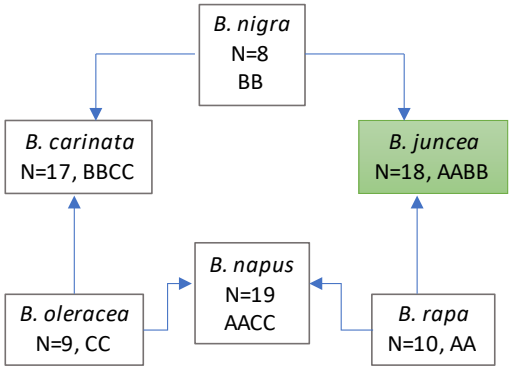

Figure 2. Genome relationships of Brassica species (simplified from OECD, 2012). Capital letters A, B, C represent the genome symbols and N is the number of chromosomes in each genome.

B. juncea species are grown for their seed oil or condiment production in Europe, the Americas, Australia and New Zealand (OECD, 2011). Mustard appears in some form as a component of the diet of individuals from Japan, Africa, India, Bangladesh, China, Korea, Italy, Nepal, Pakistan and in the Americas where leaves, stems, seeds and oil are consumed (Tian & Deng, 2020). In Canada and Europe, *B. juncea* is grown as a spring crop while it is sown as a winter crop in India, where it remains the prominent oilseed crop (Shekhawat et al, 2012). Bees are the primary pollen vector because the pollen of *B. juncea* is heavy and sticky and is not carried great distances by wind. Compared to the more widely grown canola species *B. napus* and *B. rapa*, *B. juncea* is more tolerant to heat and drought stress

(Woods *et al.*, 1991). The species does not shatter as readily as *B. napus* and so it can be straight cut or swathed and combined (Hemingway, 1995). In addition, *B. juncea* does not have the weedy characteristics of wild mustard and is less prone than *B. napus* and *B. rapa* to become a problem as a volunteer weed (CFIA).

Low glucosinolate and low erucic acid varieties have been developed for use as edible (canola quality) oil. The term 'canola' refers to cultivars of *B. napus, B. rapa* or *B. juncea* that meet specific standards for levels of erucic acid in the oil and levels of glucosinolates in the meal. Specifically, canola-quality cultivars must yield oil low in erucic acid (<2%) and air-dried, oil-free meal low in glucosinolates (<µ30 mol/g) (OECD 2011; Canola Council of Canada 2018, CFIA, 2017).

The *B. juncea* cultivars used for DPA transformation are canola quality. DPA juncea oil can be used as an ingredient for human nutrition and medical foods.

2 Genotype of DPA juncea

A. Origin and functions of the inserted genes

The subject of this request is for review of:

- *B. juncea* line, NUBJ1207, transformed with the already deregulated construct pJP3416_GA7-ModB (**Figure 3**) and for,
- pJP3662 an additional construct for future transformation.

Both contain the introduced genes for production of DPA in *B. juncea*. This section describes the source, vector location, and function of the genes.

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Figure 3. Map of vector **pJP3416_GA7-ModB [** **]. CBI-del**

The gene cassettes were synthesized for *Brassica* species and intentionally matched to the amino acid sequence of the native genes from the source organisms; these cassettes were also used to develop the DPA construct pJP3662. The DPA fatty acid in DPA juncea is identical to the DPA expressed in other deregulated events, such as Nuseed's DHA canola (17-236-01p) and BASF's LBFLFK event (17-321-01p) as well as DPA naturally occurring in marine sources (i.e., fish, algae). A summary of pJP3416_GA7-ModB and pJP3662 constructs, gene expression cassettes and their functions are provided in **Table 1**.

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B. NUBJ1207 event transformed with pJP3416_GA7-ModB construct.

DPA juncea was developed using *Agrobacterium*-mediated transformation of a conventional canola-quality (low erucic acid, low glucosinolates) *B. juncea* cultivar, JT005 (Burton et al., 2007) to introduce the biosynthetic pathway for production of long chain omega-3 fatty acids (Belide et al., 2021). The pJP3416_GA7-ModB construct (GA7_ModB, **Figure 3 and Figure 4A**) used for DPA juncea

production is the same vector used to produce DHA canola and has already been deregulated (17-236-01p), but with a truncation of the last gene (Pavsa-Δ4D) in the DHA biosynthesis pathway, therefore, the biosynthesis pathway ends at DPA.

Like the previously deregulated DHA canola event, NUBJ1207 expresses the glufosinate herbicide-resistant trait resulting from expression of the *pat* gene that encodes the enzyme phosphinothricin-N-acetyltransferase (PAT). The PAT enzyme works by acetylating L-phosphinothricin, which is the active isomer of glufosinate herbicides, rendering the crop resistant to application of this non-selective herbicide during the growing season. A detailed description of the genetic elements, their location, size, function and source are provided in **Table 2.**

Table 2. Description of the genetic elements of pJP3416_GA7-ModB used for DPA *juncea* transformation.
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Figure 4A shows the **[**

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4B, **Table 3)**.

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Figure 4. **A. [** **] B. [** **CBI-del**
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C. [**CBI-del**

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Table 4. Description of the genetic elements pJP3662 for *B. juncea* transformation.

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The DPA synthetic pathway therefore consists of OA being converted to LA by the desaturase Lackl-Δ12D, LA is desaturated to ALA by Picpa-ω3D, ALA to SDA by Micpu-Δ6D, SDA is converted to ETA by Pyrco-Δ6E, ETA to EPA by Pavsa-Δ5D, and finally EPA to DPA by Pyrco -Δ5E. All genes are regulated by seed-specific promoters. This pathway represents the same MOA as was used for DHA canola minus the last step of converting DPA to DHA catalyzed by Pavsa-Δ4D.

3 Sequences

A. NUBJ1207 line

As shown above in Figure 4B and Table 3, **[**

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T-DNA RB or LB

Promoter
TMV enhancer
CDS, ATG
Terminator
Spacer (MAR)

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B. pJP3662 DPA Construct

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4 Intended Trait, Mechanism of Action and Phenotype of DPA juncea

The intended trait is production of long chain omega-3 fatty acids with a significant amount of DPA in seed oil. The six newly introduced desaturase and elongase genes in the NUBJ1207 event express fatty acid enzymes of the DPA biosynthetic pathway (**Figure 1**). OA is converted to LA by the desaturase Lackl-Δ12D, LA is desaturated to ALA by Picpa-ω3D, ALA to SDA by Micpu-Δ6D, SDA is converted to ETA by Pyrco-Δ6E, ETA to EPA by Pavsa-Δ5D, and EPA to DPA by Pyrco-Δ5E. All genes are regulated by seed specific promoters. The resulting phenotype is a *B. juncea* plant that contains ω3 LC-PUFAs, *i.e.,* DPA and others in the seed oil.

As shown in **Table 5**, inserted enzyme expression in *B. juncea* seed results in the production of oil containing DPA, and other ω3 LCPUFAs with proven health benefits. NUBJ1207 has been advanced to T_6 seeds which contain about 12% DPA and about 36% of total omega-3 fatty acids (Belide et al., 2021).

Table 5. Fatty acid profile of seed oil from wild type (WT) *B. juncea* and NUBJ1207 (Belide et al., 2021).

Plant ID	C16:0	C18:0	C18:1	C18:1 d11	C18:2	C18:3 ω6	C18:3 ω3	C20:0	C18:4 ω3	C20:1 D11	C20:3 ω3	C20:4 ω3	C20:5 ω3	C22:4 ω3	C22:5 ω3-DPA	C22:6 ω3-DHA	Others
WT	4.3±0.2	2.0±0.0	38.0±0.9	3.8±0.4	33.6±0.5	-	15.3±0.8	0.6±0.0	-	1.0±0.1	-	-	-	-	-	-	1.5
DPA (T_6)	4.6±0.0	2.7±0.1	36.5±1.7	3.3±0.1	7.0±0.2	0.6±0.0	20.5±0.3	0.7±0.1	1.2±0.1	1.1±0.0	0.5±0.0	4.2±0.2	0.7±0.1	2.0±0.1	12.0±1.3	-	2

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In addition to the desaturase and elongase enzyme genes, the *pat* gene was introduced as a selectable marker to encode PAT which confers tolerance to phosphinothricin and glufosinate ammonium-based herbicides driven by a constitutive promoter. The PAT protein has been introduced in many GM crops that have been consumed without adverse effects to human or animal health.

Summary

DPA juncea is being developed to produce long chain omega-3 fatty acids with significant amounts of DPA in seed oil through genetic engineering. The set of genes were constructed to express the desaturase and elongase fatty acid enzymes of the DPA biosynthetic pathway, which together produce DPA, and other ω3 LCPUFAs with proven health benefits. This is the same pathway and enzymes used in the previously approved DHA canola event except that it does not contain the last enzyme required to produce DHA from DPA. The presence of ω3 LCPUFA in the biosynthetic pathway, ending at DPA confirms the MOA is functional in juncea seeds.

Desaturase and elongases are transmembrane proteins that exist in all types of biological membranes. They play critical roles in regulating the length and degree of unsaturation of fatty acids. Long chain omega-3 fatty acids are essential to human and animal health, DPA juncea offers a sustainable source to produce these important nutrients.

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