UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/560,654	07/27/2012	Luis Rafael Herrera-Estrella	LIP 301USCON1	5597

23581 7590 09/26/2019

KOLISCH HARTWELL, P.C.
520 SW YAMHILL STREET, SUITE 300
PORTLAND, OR 97204

EXAMINER	
BURAN, ASHLEY KATE	

ART UNIT	PAPER NUMBER
1662	

NOTIFICATION DATE	DELIVERY MODE
09/26/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docketing@khpatent.com
veronica@khpatent.com

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

Ex parte LUIS RAFAEL HERRERA-ESTRELLA,
DAMAR LISBETH LÓPEZ-ARREDONDO,
and ALFREDO HERIBERTO HERRERA-ESTRELLA

Appeal 2019-003981
Application 13/560,654
Technology Center 1600

Before ULRIKE W. JENKS, TIMOTHY G. MAJORS, and MICHAEL A. VALEK, *Administrative Patent Judges*.

VALEK, *Administrative Patent Judge.*

DECISION ON APPEAL

Pursuant to 35 U.S.C. § 134(a), Appellant submits this appeal under 35 U.S.C. § 134(a) involving claims to a method of producing a substance of interest. Examiner rejected claims 1, 2, 4, 6–11, 18, 21, and 24–29 for lack of written description and claims 1, 2, 4–11, 18, 21, and 23–29 as obvious.[1] We have jurisdiction under 35 U.S.C. § 6(b).

---

[1] We use the word "Appellant" to refer to "applicant" as defined in 37 C.F.R. § 1.42. Appellant identifies Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV) as the real party in interest. App. Br. 4. Herein, we refer to the Non-Final Action mailed March 9, 2018 ("Non-Final Act."); Appellant's Appeal Brief filed November 13, 2018 ("App. Br."); and Examiner's Answer mailed February

We AFFIRM–IN–PART, 37 CFR § 41.50(b), affirming the written description rejection and reversing the obviousness rejection, and enter a New Ground of Rejection for claims 1, 5, 18, and 23 under 35 U.S.C. § 103.

STATEMENT OF THE CASE

Claims 1, 2, 4–11, 18, 21, and 23–29 are on appeal and can be found in the Claims Appendix of the Appeal Brief. App. Br. 41–44. Claims 1 and 18 are illustrative. They read as follows:

> 1. A method of producing a substance of interest, comprising:
>
> providing an algae transgenically adapted (a) to synthesize the substance of interest and (b) to express a phosphite dehydrogenase enzyme that catalyzes oxidation of phosphite to phosphate, the enzyme being present at a level sufficient to enable the algae to grow using phosphite as a source of phosphorus; and
>
> growing the algae in a medium containing phosphite.
>
> 18. A method of producing a substance of interest, comprising:
>
> growing a photosynthetic cell in a medium containing phosphite, the cell being transgenically adapted to synthesize the substance of interest and to grow using phosphite as a source of phosphorus;
>
> wherein the cell is transgenically adapted to express a phosphite dehydrogenase enzyme that catalyzes oxidation of phosphite to phosphate.

App. Br. 41–42 (Claims Appendix). Claim 5 depends from claim 1 and additionally specifies that the "phosphite dehydrogenase is PtxD of Pseudomonas stutzeri, an analog or derivative of PtxD, or a PtxD-like

---

26, 2019 ("Ans.").

homolog."[2] Claim 23 depends from claim 18 and recites the same limitation as in claim 5.

Examiner rejected claims 1, 2, 4, 6–11, 18, 21, and 24–29 under 35 U.S.C. § 112(a) for failing to comply with the written description requirement ("the Written Description Rejection") and claims 1, 2, 4–11, 18, 21, and 23–29 under 35 U.S.C. § 103 as unpatentable over Abad,[3] in view of Metcalf,[4] Hallman,[5] Kimura,[6] Borowitzka,[7] and Metcalf 98 ("the Obviousness Rejection").[8] Non-Final Act. 3–12. Appellant seeks review of the Obviousness Rejection, but "does not request review" of the Written Description Rejection. App. Br. 10. Accordingly, we summarily affirm the Written Description Rejection. *See* MPEP § 1205.02 ("If a ground of rejection stated by the examiner is not addressed in the appellant's brief, appellant has waived any challenge to that ground of rejection and the Board

[2] The Specification indicates that PtxD is a phosphate dehydrogenase enzyme with the sequence in SEQ ID NO: 1. Spec. 16, l. 4, 47, ll. 4–6.
[3] Mark Scott Abad et al., US 2007/0124833 A1; published May 31, 2007 ("Abad").
[4] William W. Metcalf et al., US 2004/0091985 A1; published May 13, 2004 ("Metcalf").
[5] Armin Hallman, *Algal Transgenics and Biotechnology*, Transgenic Plant Journal, Vol. 1, 81–98 (2007) ("Hallman").
[6] Ryoji Kimura et al., EP 0 635 209 B1; published May 17, 2000 ("Kimura").
[7] Michael A. Borowitzka, *Commerical Production of Microalgae: ponds, tanks, tube, and fermenters*, Journal of Biotechnology, Vol. 70, 313–321 (1999) ("Borowitzka").
[8] William W. Metcalf et al.; *Molecular Genetic Analysis of Phosphite and Hypophosphite Oxidation by Pseudomanas stutzeriWM88*, Journal of Bacteriology, Vol. 180, 5547–5558 (1998) ("Metcalf 98").

may summarily sustain it, unless the examiner subsequently withdrew the rejection in the examiner's answer.").

With respect to the Obviousness Rejection, the issue is: has Examiner established a prima facie case of obviousness, and if so has Appellant provided evidence of secondary considerations that, upon reweighing the entire merits of the case, demonstrates Examiner's determination of obviousness is not supported by the preponderance of the evidence of record?

Findings of Fact

FF1. Abad teaches transgenic plants with "enhanced agronomic traits" that have been transformed to "express a protein having [an] amino acid sequence with at least 90% identity" to a specified set of "consensus amino acid sequences" and "homologs thereof listed in Table 2." Abad ¶ 7. SEQ ID NO: 20228 is one of the homolog sequences identified in Abad. *Id.* ¶ 3

FF2. Metcalf teaches the amino acid sequence for a phosphite dehydrogenase enzyme designated PtxD, which "was cloned from Pseudomonas and also found in other bacteria."[9] Metcalf, Abstr. According to Metcalf, PtxD catalyzes the oxidation of phosphite to phosphate. *Id.* Abstr., ¶ 65. Metcalf further teaches *E. coli* can be transformed "by use of a recombinant system" to overproduce PtxD and then grown on a media with phosphite as the sole source of phosphorus. Metcalf ¶¶ 65, 68, 135–136.

FF3. Hallman teaches that transgenic algae have been used to produce a variety of compounds of interest, including "recombinant antibodies,

[9] Examiner determines that the SEQ IDs taught in Metcalf share "100% identity" with SEQ ID NO:1 of Appellant's Specification. Non-Final Act. 7. Appellant does not challenge that finding on appeal.

vaccines, insecticidal proteins, or bio-hydrogen." Hallman, Abstr. Hallman further teaches that "[s]electable marker genes, promoters, reporter genes, transformation techniques, and other genetic tools and methods" are available for "various species" of algae. *Id.*

FF4. Hallman teaches that algae are "plants" and while they "never have true stems, roots and leaves . . . they are normally capable of photosynthesis." Hallman 82. The teachings in Hallman "mainly" concern "(eukaryotic) microalgae" as opposed to "prokaryotic algae (Cyanobacteria)." *Id.* at 83.

FF5. Hallman describes techniques for transforming algae, including the use of selectable marker genes. *See* Hallman 90–91. According to Hallman, "[t]he use of selectable marker genes is normally required in all experiments that aim to generate stable transgenic algae, since only a very low percentage of treated organisms are successfully transformed." *Id.* at 91. One example of a selectable marker gene that Hallman teaches is the "nitrate reductase gene (*nit*)." *Id.* According to Hallman, "[b]y using this gene, former *nit* [defective] mutants gain the trait to utilize nitrate as the sole nitrogen source in transformation experiments." *Id.*

FF6. Metcalf 98 teaches that because "[a]ll organisms require P [i.e., phosphorus] in its most-oxidized form, phosphate, for growth . . . . it is possible to select for organisms capable of oxidizing reduced P compounds to phosphate." Metcalf 98, 5550. According to Metcalf 98, "in media containing reduced P compounds," such as phosphite, "as the sole P source, only those organisms capable of oxidizing these reduced compounds to phosphate are able to grow." *Id.*

FF7. Borowitzka teaches that "[a] common feature of most of the algal species currently produced commercially . . . is that they grow in highly

selective environments which means that they can be grown in open air cultures and still remain relatively free of contamination by other algae and protozoa." Borowitzka, 315. According to Borowitzka, "[t]hose species which do not have this selective advantage must be grown in closed systems," which are more costly than open-air systems. *See id.* at 319 ("The challenge now is to reduce the construction costs of these [closed] systems further to make them more economically competitive.").

ANALYSIS

Examiner determines that Abad teaches the transformation of plants with a construct comprising "a sequence (SEQ ID NO: 20228) that shares 100% identity with" SEQ ID NO: 1, i.e., PtxD of Pseudomonas stutzeri. *See* Non-Final Act. 7; Ans. 9. Examiner acknowledges that Abad does not teach that the enzyme comprising SEQ ID NO: 20228 "catalyzes the oxidation of phosphite to phosphate," but points to Metcalf's teaching that PtxD "catalyzes the oxidation of phosphite to phosphate" as evidence that PtxD's function was known in the art. Non-Final Act. 7–8. Examiner finds that Hallman teaches that algae are plants and "[m]ethods for transforming alga[e]" to produce various substances of interest "were well known and routine in the art." *Id.* at 8–9. In particular, Examiner determines that Hallman teaches that selectable marker genes are required to generate transgenic algae and that *nit*, which confers the ability to "utilize nitrate as the sole nitrogen source," is described as an example of such. *Id.* at 9 (quoting Hallman 91). Examiner finds that Metcalf teaches that "phosphite can be used as a selectable marker" and "only organisms that can oxidize phosphite will grow in media where phosphite is sole source of phosphorus,"

which is a "similar[]" concept to the *nit* "mutants taught by Hallman." *Id.* at 10 (emphasis omitted). According to Examiner, one of skill in the art would understand from Kimura that "algae do not readily utilize phosphite as a source of phosphorus." *Id.* at 9. Thus, Examiner concludes that it would be obvious to use the PtxD gene as a selectable marker gene in algae transformed to express a substance of interest and to take advantage of the benefits of open-air systems, as taught in Borowitzka, by growing them in selective "phosphite containing environments." *See id.* at 9–11.

Appellant contends that the Obviousness Rejection fails for several reasons. First, while conceding that Abad's SEQ ID NO: 20228 is the sequence for PtxD, Appellant urges that "Abad does not assign a name or function to the sequence" and merely refers to it as a "homolog" of "another sequence . . . which improves cold germination tolerance, heat stress tolerance, high salinity stress tolerance, and osmotic stress tolerance in transformed Arabidopsis." App. Br. 20 (citing Abad ¶¶ 92, 94–96, Table 3) (emphasis omitted). Thus, argues Appellant, "there is nothing in Abad that would motivate the skilled person to single out SEQ ID NO:20228 from Abad's 30,000 homologs . . . to create an algae or photosynthetic cell expressing that sequence and to grow it in a medium containing phosphite." *Id.* at 20–21. Second, Appellant contends that the rejection is improper because the proposed modification of Abad (i.e., to transform an algae or photosynthetic cell to express PtxD) changes Abad's "principle of operation," which is enhancing an "<u>existing</u> plant trait"—not conferring a "<u>new</u> trait" such as phosphite oxidation/metabolism. *Id.* at 21–22. Third, Appellant argues there is no prima facie case because "[t]here is nothing in the record to show that [untransformed] algae cannot utilize phosphite as

their phosphate source" and that Examiner has misinterpreted Kimura. *Id.* at 22–24. Fourth, Appellant contends there is "[c]ompelling evidence for secondary considerations of non-obviousness presented in the Herrera Declaration," including unexpected results as well as industry praise and commercial interest. *See Id.* at 24–39 (citing Declaration of Luis Rafael Herrera-Estrella dated July 24, 2017 ("Herrera-Estrella Decl.")).

Upon considering the record as a whole, we are persuaded by Appellant's third argument. *See* App. Br. 22–24. In particular, Kimura does not support Examiner's finding that one of ordinary skill in the art would know that algae and other photosynthetic cells, e.g., plant cells, [10] are unable to utilize "phosphite" as that term is used in Appellant's claims. *See* Non-Final Act. 9. Kimura teaches that certain organophosphite and organophosphate compounds are "antifouling agent[s]" that reduce "adhesion of aquatic pests," such as algae, to submerged structures. *See* Kimura ¶¶ 1–2, 13. The Specification, however, defines "phosphite" to be inorganic phosphite, that is, "three oxygens and one hydrogen . . . bonded directly to a phosphorus atom" and specifically distinguishes "phosphite" from "'organophosite,' which is an organic version of phosphite in which one or more of the phosphite oxygens are bonded to organic moieties." Spec. 14, ll. 1–12 (definition of "phosphite"). Thus, the term "phosphite" in Appellant's claims is limited to inorganic phosphite. *See Phillips v. AWH Corp.*, 415 F.3d 1303, 1316 (Fed. Cir. 2005) (en banc) ("[T]he specification

[10] Appellant does not dispute Examiner's finding that the "algae" recited in claim 1 are, in fact, plants, nor does Appellant dispute that the "photosynthetic cell" in claim 18 is "inclusive of plants and plant cells." Non-Final Act. 6; Ans. 3.

may reveal a special definition given to a claim term by the patentee" that "governs" the construction of that term). There is, however, no teaching in Kimura that inorganic phosphite is an antifouling agent or that otherwise suggests plants such as algae cannot utilize phosphite as a source of phosphorus.

Examiner's prima facie case is premised on a finding that algae/plants cannot utilize phosphite. *See* Non-Final Act. 9–10 (finding "[o]ne would have been motivated to generate transgenic algae that express the PtxD enzyme because it was known in the art that (i) algae, like the land plants taught by Abad et al, are photosynthetic organisms that do not readily metabolize phosphite as a source of phosphorous"); *see also* Ans. 8, 16, 23–24. Kimura does not support that finding. However, we agree with Examiner's conclusion that Appellant's claims would be obvious if the prior art, in fact, demonstrated that plants do not readily utilize phosphite as a source of phosphorus. Thus, while we reverse the Obviousness Rejection, we do so solely because Kimura does not support such a finding. We find Appellant's other arguments unpersuasive as explained below.

New Ground of Rejection

We enter a new ground rejecting claims 1, 5, 18, and 23 under 35 U.S.C. § 103 over Abad, Metcalf, Hallman, Borowitzka, Metcalf 1998, and McDonald.[11] McDonald teaches that plants are "clearly unable to substitute the [phosphorous] in [phosphite] for the [phosphorous] in [phosphate]."[12]

---

[11] Allison E. McDonald et al., *Phosphite (Phosphorous Acid): Its Relevance in the Environment and Agriculutre and Influence on Plant Phosphate Starvation Response*, J. Plant Nutrition, Vol. 24, 1505–1519 (2001) ("McDonald").

[12] McDonald refers to phosphite as "Phi," which is inorganic phosphite, as

McDonald, 1514. "As plants are unable to metabolize [phosphite], it persists in tissues for extensive periods" and causes deleterious effects in phosphate-starved plants. *Id.* at 1512. Accordingly, McDonald teaches that plants, which would include the algae and photosynthetic cells recited in claims 1, 5, 18, and 23, cannot readily utilize phosphite as a phosphorous source.

We combine this teaching from McDonald with Examiner's prior findings regarding Abad, Metcalf, Hallman, Borowitzka, and Metcalf 1998 as set forth on pages 7–12 of the Non-Final Act and pages 4–13 of the Answer, which we agree with (*see* FF1–FF7) and incorporate by reference herein. With the substitution of McDonald's teaching that plants "do not readily metabolize phosphite as a source of phosphorous" for Kimura, we agree with Examiner's conclusion that at least claims 1, 5, 18, and 23 are obvious over these references.[13] *Id.* In particular, we agree with Examiner's determination that:

> One would have been motivated to generate transgenic algae that express the PtxD enzyme because it was known in the art that (i) algae, like the land plants taught by Abad et al. are photosynthetic organisms that do not readily metabolize phosphite as a source of phosphorus, (ii) overexpression of PtxD in an organism confers the property of phosphite

---

recited in Appellant's claims. *See* McDonald, 1508.

[13] Should there be further prosecution, we leave to Examiner to determine whether to make a new ground of rejection of any other claims. *See* 37 C.F.R. § 41.50(b); s*ee also* Manual of Patent Examining Procedure (MPEP) § 1213.02. Under 37 C.F.R. § 41.50(b), the Board may, in its decision, make a new rejection of one or more of any of the claims pending in the case. Because the exercise of authority under 37 C.F.R. § 41.50(b) is discretionary, no inference should be drawn from the decision to exercise that discretion with respect to some but not all of the claims on appeal.

> oxidation thus allowing said organism to utilize phosphite as a source of phosphorous, and (iii) phosphite is an effective selectable marker . . . wherein only the organisms that express the phosphite dehydrogenase enzyme are able to grow in phosphite containing environments.

Non-Final Act. 10–11. Moreover, a skilled artisan would reasonably expect that the PtxD gene could be successfully used as a selectable marker gene for algae transformed to synthesize a compound of interest because: (1) Metcalf 1998 teaches that it is possible to select for any organism based on its capability to oxidize phosphite to phosphate; (2) Metcalf reports that the same transformation successfully conferred the ability to utilize phosphite in *E. coli*; and (3) Hallman describes transformation with a gene that operates similarly to the PtxD gene, i.e., by altering the organism's nutritional requirements, as a selectable marker gene for transgenic algae. *See* FF2, FF5, FF6.

We are not persuaded by Appellant's arguments that Abad's SEQ ID NO: 20228 is "buried" in that reference such that it would not be obvious to transform algae to express that sequence. *See* App. Br. 19–21. Abad teaches the transformation of plants to express proteins corresponding to specific sequences—one of which (SEQ ID NO: 20228) is the sequence for PtxD. FF1. The fact that a reference discloses "a multitude of effective combinations does not" in and of itself "render any particular [one] less obvious." *Merck & Co., Inc. v. Biocraft Labs., Inc.*, 874 F.2d 804, 807 (Fed. Cir. 1989). While it is true, as Appellant urges, that Abad does not teach that PtxD catalyzes the oxidation of phosphite to phosphate, Metcalf does. FF2. Indeed, Metcalf teaches that transforming *E. coli* to express PtxD altered its nutritional requirements, allowing the transformed organism to be

grown on a medium where phosphite is the sole source of phosphorus. *Id.* Moreover, Hallman teaches that selectable marker genes for algae include genes that alter the transformed organism's nutritional requirements. FF5. Thus, even if one accepts Appellant's argument that Abad's SEQ ID NO: 20228 is "buried in a listing of over 30,000 homologs," (App. Br. 20) the other references demonstrate that it would be obvious to select that particular sequence to transform algae and photosynthetic cells.

We are also unpersuaded by Appellant's argument that such a modification would impermissibly change Abad's "principle of operation." *See* App. Br. 21–22. Abad describes "genes and uses for plant improvement." Abad (title). According to Abad, such "improvement" in some "aspects of the invention" involves transforming a plant to "express[] a protein that provides tolerance from exposure to an herbicide applied at levels that are lethal to a wild type of said plant." *Id.* ¶ 9. Such a modification is similar to the modification proposed in the obviousness rejection here. It is immaterial whether one describes this as conferring a "new trait" or merely enhancing an "existing" one. *See* App. Br. 21. Put differently, the distinction Appellant attempts to draw regarding Abad's "principle of operation" is one of semantics. *Id.* It does not reflect a change to scientific or technical principles underlying Abad's teaching that plants can be improved by transforming them to express the sequences taught therein.

Finally, we are unpersuaded by Appellant's arguments based on the Herrera-Estrella Declaration. Appellant contends that the "use of phosphite as fertilizer and contaminant suppressant is an unexpected product of modifying algae to allow them to use phosphite instead of phosphate as a

phosphorus source." *See* App. Br. 35. But the record supports that a skilled artisan would expect such results from the prior art. *In re Skoner*, 517 F.2d 947, 950 (CCPA 1975) (holding that *expected* beneficial results are evidence of obviousness). Indeed, Metcalf teaches that an organism can be transformed with the PtxD gene, thereby allowing it to be grown in a medium containing phosphite as the sole phosphorus source. FF1. Accordingly, it would be expected from the prior art that algae transformed to express PtxD could be fertilized with phosphite. In addition, McDonald teaches that plants cannot effectively metabolize phosphite (*see* McDonald 1512) and Hallman teaches that genes that alter a transformed algae's nutritional requirements are effective to select for the transformed algae (FF5). Thus, a skilled artisan would expect phosphite to suppress contaminants, e.g., other untransformed algae, in algae transformed to express PtxD, particularly in a low-phosphate environment. *See In re Baxter Travenol Labs.*, 952 F.2d 388, 392 (Fed. Cir. 1991) ("[W]hen unexpected results are used as evidence of nonobviousness, the results must be shown to be unexpected compared with the closest prior art.").

Appellant's arguments pertaining to commercial success and industry praise are likewise unpersuasive. *See* App. Br. 33. As we understand the evidence in and attached to the Herrera-Estrella Declaration, the alleged commercial interest and industry praise relates to engineering crops so that such crops can be fertilized by phosphite as opposed phosphate.[14] Appellant has not clearly established a nexus between that evidence and the "method

[14] Our review is limited by the fact that some of the exhibits attached to the Herrera-Estrella Declaration are not in English. *See* Herrera-Estrella Decl. Exs. B and D. Appellant has not provided translations of those exhibits.

of producing a substance of interest" in claims 1, 5, 18, and 23. *See In re GPAC Inc.*, 57 F.3d 1573, 1580 (Fed. Cir. 1995) (citation omitted) ("For objective evidence to be accorded substantial weight, its proponent must establish a nexus between the evidence and the merits of the claimed invention."). Indeed, these claims encompass the use of the PtxD gene as a selectable marker to select for algae and photosynthetic cells transformed to express a substance of interest in laboratory research. Thus, Appellant's arguments regarding a need for alternatives to phosphate fertilizers in commercial agriculture and avoiding algae bloom from runoff of such fertilizers, as well as the alleged "professional and commercial interest" that Appellant's research has garnered is not reasonably commensurate with the broader scope of claims 1, 5, 18, and 23. *See In re Harris*, 409 F.3d 1339, 1344 (Fed. Cir. 2005) (Evidence of unexpected results must also be "commensurate in scope with the degree of protection sought by the claim[s]" on appeal to demonstrate non-obviousness). For all these reasons, the Herrera-Estrella Declaration is insufficient to overcome the new ground of rejection.

CONCLUSION

In summary:

Claims Rejected	Basis	Affirmed	Reversed	New Ground
1, 2, 4, 6–11, 18, 21, 24–29	§ 112(a)	1, 2, 4, 6–11, 18, 21, 24–29		
1, 2, 4–11, 18, 21, 23–29	§ 103 Abad, Metcalf, Hallman, Kimura, Borowitzka, Metcalf 98		1, 2, 4–11, 18, 21, 23–29	
1, 5, 18, 23	§ 103 Abad, Metcalf, Hallman, McDonald, Borowitzka, Metcalf 98			1, 5, 18, 23
Overall Outcome		1, 2, 4, 6–11, 18, 21, 24–29	5, 23	1, 5, 18, 23

FINALITY AND RESPONSE

This decision contains a new ground of rejection pursuant to 37 C.F.R. § 41.50(b). 37 C.F.R. § 41.50(b) provides "[a] new ground of rejection pursuant to this paragraph shall not be considered final for judicial review."

37 C.F.R. § 41.50(b) also provides that the Appellant, WITHIN TWO MONTHS FROM THE DATE OF THE DECISION, must exercise one of the following two options with respect to the new ground of rejection to avoid termination of the appeal as to the rejected claims:

> (1) *Reopen prosecution*. Submit an appropriate amendment of the claims so rejected or new evidence relating to the claims so rejected, or both, and have the matter reconsidered by the examiner, in which event the proceeding will be remanded to the examiner. . . .

> (2) *Request rehearing*. Request that the proceeding be reheard under § 41.52 by the Board upon the same record. . . .

AFFIRMED-IN-PART; 37 C.F.R. 41.50(b)

Save Form

Notice of References Cited	Application/Control No. 13/560,654	Applicant(s)/Patent Under Patent Appeal No. 2019-003981	
	Examiner	Art Unit 1662	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Journal of Plant Nutrition (2001) - McDonald
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

JOURNAL OF PLANT NUTRITION, 24(10), 1505–1519 (2001)

PHOSPHITE (PHOSPHOROUS ACID): ITS RELEVANCE IN THE ENVIRONMENT AND AGRICULTURE AND INFLUENCE ON PLANT PHOSPHATE STARVATION RESPONSE

Allison E. McDonald, Bruce R. Grant, and William C. Plaxton*

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

ABSTRACT

Phosphites ($H_2PO_3^-$; Phi) are alkali metal salts of phosphorous acid [$HPO(OH)_2$] that are being widely marketed either as an agricultural fungicide or as a superior source of plant phosphorus (P) nutrition. Published research conclusively indicates that Phi functions as an effective control agent for a number of crop diseases caused by various species of pathogenic pseudo fungi belonging to the genus *Phytophthora*. However, evidence that Phi can be directly used by plants as a sole source of nutritional P is lacking. When Phi is administered in such as way as to allow it to come into contact with bacteria, either associated with plant root systems or in the soil, then the oxidation of Phi to phosphate (HPO_4^{2-}; Pi) may take place. By this indirect

*Corresponding author. Fax: 1-613-533-6617; E-mail: plaxton@biology.queensu.ca

method Phi could thus become available to the plant as a P nutrient. The rates at which this occurs are slow, taking months or as much as a year, depending on the soil type. Phi is not without direct effects on plants itself, as Phi concentrations comparable to those required to control plant infection by pathogenic *Phytophthora*, or to restrict *Phytophthora* growth in sterile culture, are extremely phytotoxic to Pi-deprived, but not Pi-fertilized, plants. This is because Phi treatment negates the acclimation of plants to Pi deficiency by disrupting the induction of enzymes (e.g., acid phosphatase) and transporters (e.g., high-affinity plasmalemma Pi translocator) characteristic of their Pi starvation response. Thus, Phi intensifies the deleterious effects of P-deficiency by 'tricking' Pi-deprived plant cells into sensing that they are Pi-sufficient, when, in fact, their cellular Pi content is extremely low. The Phi anion appears to effectively obstruct the signal transduction pathway by which plants (and yeast) perceive and respond to Pi deprivation at the molecular level. The review concludes by citing concerns and recommendations regarding the significant input of Phi into food products and the environment that arises from its extensive use in agriculture and industry.

INTRODUCTION

Phosphorus (P) is one of the major elements required by all living species to grow and develop. Phosphorus does not naturally occur as the free element because it is too reactive, combining rapidly with other elements such as oxygen or hydrogen. A global P cycle occurs by the oxidation and reduction of P compounds by electron transfer reactions (Fig. 1). Although bacteria have been implicated in the redox reactions of P (1–4), the biochemical mechanism and genetics of these transformations are not well understood. When P is oxidized to the fullest extent possible, the product is orthophosphate (PO_4^{3-}; Pi), in which four oxygen atoms have bonded with a single P atom. At neutral pH the Pi ion is present as a mixture of HPO_4^{2-} and $H_2PO_4^-$. It is as form $H_2PO_4^-$ that Pi is normally transported into plant cells. Pi is intimately involved with cellular bioenergetics and metabolic regulation, and is also an important structural component of macromolecules such as nucleic acids and phospholipids. It plays a critical role in virtually all major metabolic processes in plants, including photosynthesis and respiration. Unlike some bacterial cells (1–4), Pi cannot be reduced within the plant cell to a lower oxidation state. Rather, Pi is either sequestered in the cell vacuole or incorporated into organic form (e.g., initially as

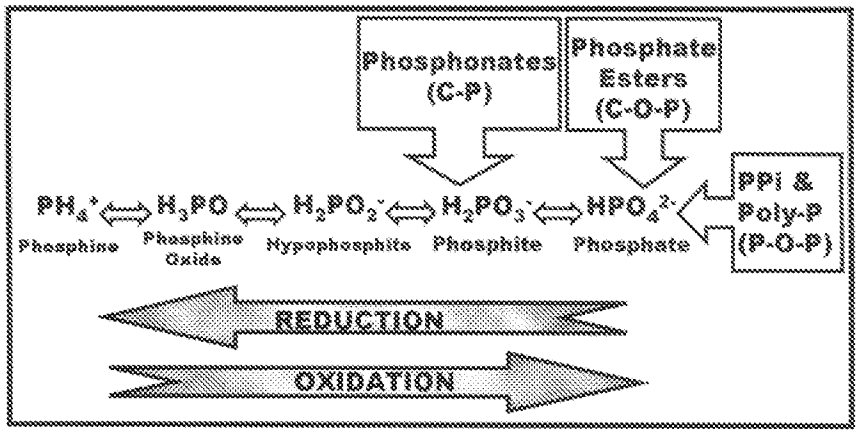


Figure 1. Natural P cycle that is believed to exist in various soil dwelling microbes. Adapted from Ohtake et al. (2).

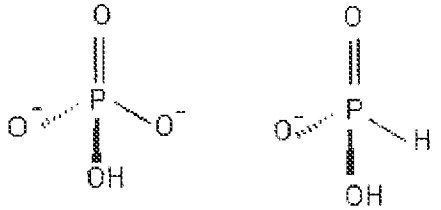
ATP) via photo- or oxidative phosphorylation. Phosphorlysis by Pi of certain 'high energy' ester bonds by enzymes such as starch phosphorylase also results in the direct covalent incorporation of Pi into organic compounds (5).

Despite its ubiquitous importance to plant metabolism, Pi is one of the least available nutrients in many aquatic and terrestrial ecosystems. Most of the Pi in the earth's crust occurs in an insoluble mineral form that is largely unavailable to plants (5). The massive use of Pi in fertilizers, accounting for 90% of mineral Pi use worldwide, demonstrates how the free Pi levels of most soils are suboptimal for plant growth. It is widely accepted that Pi is the sole P-containing nutrient important for optimal plant growth and development (5). However, over the past 20 years a reduced form of Pi known as phosphite[1] ($H_2PO_3^-$; Phi) has increasingly been used to improve the yield of many crop species. The extensive use of Phi and its related products in agriculture has raised considerable controversy in the scientific world. The aim of this review is to provide an objective summary of Phi's chemistry and biology, with a focus on its applications in agriculture.

[1]Although the terms phosphite and phosphonate have both been used to describe salts of phosphorous acid, $HPO(OH)_2$, phosphonate is also employed for the nomenclature of compounds containing a C-P bond (5). To avoid ambiguity this review therefore uses the term phosphite for the description of alkali metal salts of phosphorous acid.

The Chemistry of Phosphate Versus Phosphite

Phosphite differs from Pi such that in Phi, an oxygen atom is replaced by a hydrogen (Fig. 2). This substitution results in profound differences in the manner in which the two compounds behave in living organisms. In Pi, the P atom sits at the center of a tetrahedron, with the oxygen atoms distributed at the points of the tetrahedron (Fig. 2). The charge on the ion is distributed evenly among these four oxygen atoms so that the entire structure is wholly symmetrical from whatever face the tetrahedron is viewed. In Phi, the arrangement of the P atom is also at the center of a tetrahedron, but the perfect symmetry that is a feature of the Pi ion, is lost. For Pi to react and take part in the biochemistry of living organisms, it must interact with enzymes, the catalysts for the cell's chemical reactions which collectively constitute metabolism. It appears that enzyme Pi binding sites recognize three of the four oxygen atoms, and bind the Pi ion on the enzyme surface. Both the shape of the molecule and the charge distribution seem to influence this binding. Once Pi has bound to the enzyme, the remaining oxygen will protrude from the surface, and thus becomes available to react with other molecules in the reaction catalyzed by the enzyme. Phi has only one face of the tetrahedron relatively similar to all the faces of the Pi tetrahedron, so if it is to bind to the surface of an enzyme that normally binds Pi, it must bind at this face. When Phi binds to the enzyme surface in this orientation, it is the hydrogen atom bonded to the P atom that protrudes from the enzyme surface, not an oxygen atom as in Pi. Thus, Phi cannot enter into the same biochemistry as Pi. Owing to this, as well as the difference in charge distribution on the two anions, most enzymes involved with phosphoryl transfer reactions readily discriminate between Phi and Pi (5). Likewise, Phi is a very poor *in vitro* effector of *Brassica nigra* (black mustard) PPi-dependent



Pi (HPO_4^{2-}) **Phi** ($H_2PO_3^-$)

Figure 2. A comparison of the phosphate (Pi) and phosphite (Phi) anions.

phosphofructokinase and acid phosphatase (APase), relative to their potent inhibitor Pi (6). However, as discussed below, plant and yeast proteins that appear to 'recognize' Phi as Pi include plasmalemma Pi transporters, as well as the 'Pi sensing machinery' that allows plants and yeast to detect and respond to cellular Pi depletion at the molecular level.

Bacterial Phosphite Metabolism

The non-enzymatic oxidation of Phi to Pi in air may occur very gradually over time (7,8). However, in 1950 Adams and Conrad (1) determined that the oxidation of Phi to Pi in soil was largely due to the microbial activity within the soil. Phi metabolism initially requires the absorption and assimilation of Phi by the soil dwelling bacteria. Phi is then enzymatically oxidized to Pi before being incorporated into organic form. The oxidation of Phi serves two important purposes for these microorganisms: the production of energy and the production of Pi. Both of these products would be advantageous, especially since Pi is a limiting nutrient in many natural ecosystems. Some bacteria are capable of utilizing Phi as their sole source of P, but all bacteria studied to date preferentially utilize Pi as their P nutrient.

In recent years there have been advances made in microbial genetics that have begun to shed light on the processes used by bacteria to oxidize Phi to Pi (2–4). Phi is also an intermediate in the pathway that oxidizes hypophosphite to Pi (Fig. 1) (2). Phi can be oxidized to Pi by prokaryotes such as *Escherichia coli, Klebsiella aerogenes, Agrobacterium tumefaciens,* and several species of *Pseudomonas* and *Rhizobium*. Within the genome of *Pseudomonas stutzeri* the region required for Phi oxidation to Pi putatively encodes a binding-protein-dependent Phi transporter (3). In *E. coli*, the gene products PhnC, PhnD, and PhnE probably comprise a periplasmic binding-protein-dependent transporter capable of Phi uptake (4). Intracellular Phi is oxidized into Pi in *E. coli* and *K. aerogenes* by the gene products of the *phn* cluster (2–4). It is currently unknown what the biochemical function of these gene products are, but some are hypothesized to be lyases, transcriptional activators, dehydrogenases, and other regulatory proteins. The expression of the *E. coli phn* genes is known to be activated under conditions of Pi limitation (4). The Pi released from the oxidation of Phi apparently limits the further utilization of Phi by means of a regulatory feedback loop (2–4).

Although enzymes capable of oxidizing Phi to Pi appear to be prevalent in prokaryotes, in no case are both genetic and biochemical data available for a Phi-oxidizing system. The existence of a chromosomal region dedicated to the microbial metabolism of reduced P compounds (4) indicates that a redox cycle for P is important in the metabolism of this compound by microbes.

The Use of Phosphite in Agriculture

In the 1930s, studies were carried out using a variety of different P-containing compounds to determine their effectiveness as a means of supplying P to support plant growth. Phi was determined to be a very poor source of nutritional P, as the conversion of Phi to Pi in the soil was too slow to be agriculturally relevant (8,9). Crops grown in soils to which Phi had been added to supplement natural levels of Pi grew much more poorly than those grown on soils fertilized with Pi. In some cases, when crops were replanted in the same soils a year after the initial application of Phi, they did better than crops planted in the year of the application. This was due to the slow conversion of Phi to Pi in the soil (8). However, the increase in yield was never equivalent to that observed when crop P requirements were supplied directly as Pi. These results, together with the fact that Phi is a much more expensive way to provide P than is Pi in the form of superphosphate, would, one might expect, have permanently eliminated Phi from the interest of crop producers. However, Phi returned to the agricultural stage in the 1970s when it was shown that Phi, when reacted with ethanol to form ethyl-phosphonate, effectively suppressed several soil-borne plant diseases caused by pseudo fungi belonging to the order Oomycetes, particularly *Phytophthora* sp. (9–17). The genus *Phytophthora* are collectively responsible for many important plant diseases, of which the Irish potato blight (caused by *P. infestans*) that precipitated the Irish potato famine of 1846, is perhaps the best known. Ethyl-phosphonate is now widely marketed under the trade-name Aliette® or Fosetyl-Al. The Al part of the name stems from the use of aluminum ions (Al^{3+}) to neutralize the single charge on the ethyl-phosphonate ion, so that Fosetyl-Al has three ethyl-phosphonate ions that are ionically bonded to a single Al ion. It is Phi, released in the plant by hydrolysis of ethyl-phosphonate, that is responsible for protection of plants against the fungal pathogen (9–12). The potassium salt of Phi is an equally effective agent to control plant infection by *Phytophthora* sp. (9–15). Thus, both K-Phi and Fosetyl-Al continue to be widely employed throughout the world to control a spectrum of crop diseases brought about by pathogenic *Phytophthora* sp.

That the primary site of Phi's fungicidal action is within the fungal pathogen and not the host plant (10,11) was corroborated by the observation that 0.1 to 3 mM Phi markedly inhibited the growth of *Phytophthora* mycelia in sterile culture (11–15). ^{31}P-NMR spectroscopy revealed that Phi perturbs P metabolism in *Phytophthora* by causing a massive accumulation of polyphosphate (poly-P) and pyrophosphate (PPi) (9,14,15). Phi's toxicity in *Phytophthora* has therefore been proposed to largely arise from its capacity to increase PPi and hence indirectly inhibit key pyrophosphorylase reactions essential to anabolism (15). Phi's effectiveness in suppressing *Phytophthora* depended to some extent upon the concentration of Pi that was present (12). This was explained when it was shown that Pi and Phi ions compete for the same transporters in *Phytophthora*

and that Pi is a better competitor for these sites than Phi (13). Relatively high concentrations of Phi also inhibited the activities of several enzymes of the glycolytic and oxidative pentose-phosphate pathways in clarified *Phytophthora* extracts (16). This supports the hypothesis that Phi may inhibit several enzymes rather than acting at a single unique site within *Phytophthora*. At present there is wide agreement that these direct deleterious effects of Phi on *Phytophthora* metabolism are important in controlling the diseases which it causes in plants. However, this may not be the only means by which control is exerted (9,17,18).

Plants have evolved many effective and highly sophisticated endogenous mechanisms for combating pathogenic infections. They are able to recognize most invading organisms and respond to their presence by generating a powerful antimicrobial environment in the immediate neighborhood of the attempted invasion. This may result in the invading organism being restricted to a small part of the plant. Phi-treated plants appear to be able to generate an antimicrobial environment more effectively than those not treated with the chemical (9,17). There is a close relationship between the concentration of Phi present at the invasion site and the extent to which plant defense genes are expressed (18). Thus, it has been argued that Phi's ability to control pathogenic *Phytophthora* sp. results from an influence on the plant itself, making it able to respond more effectively to the invading organism. Others have maintained that Phi has no effect on plants, but in addition to directly restricting the growth of the fungal pathogen, Phi forces it to alter its structure in such a way that it is better recognized as an invader by the host plant. A more efficient recognition process allows a more rapid and hence more effective defense response. A recent paper (18) appears to have reconciled these two hypotheses. Studies on *Eucalyptus marginata* inoculated with *P. cinnamomi* showed that the effect of Phi in controlling the pathogen is determined by the Phi concentration at the host-pathogen interface. When Phi concentrations in the roots were low, Phi interacted with the pathogen at the site of ingress to stimulate host defense enzymes (18). When Phi concentrations in the roots were elevated, the host defenses remain unchanged, and Phi appeared to act directly on the pathogen to inhibit its growth before it was able to establish an association with the host.

Influence of Phosphite on Plant and Yeast Phosphate Starvation Responses

Regardless of the mechanism by which Phi acts to restrict *Phytophthora* during its invasion of plants, recent work has revealed that Phi does have direct effects on plants, regardless of whether they have been challenged by *Phytophthora* or not. Plants treated with Fosetyl-Al or Phi rapidly amass Phi within their cells (6,11,19,20). Phi is phloem mobile and accumulates in sink

tissues (6,9). As plants are unable to metabolize Phi (6,9,19,20), it persists in tissues for extensive periods. Nevertheless, it has been generally assumed that Phi levels used to control pathogenic *Phytophthora* do not interfere with the growth or metabolism of the host plants (8). However, recent studies demonstrated that relatively low (e.g., 1–2 mM) Phi concentrations drastically disrupt the development of Pi-starved, but not Pi-fertilized *B. nigra* seedlings, and *B. napus* (oilseed rape or canola) suspension cells (Fig. 3) (6,19). ^{31}P-NMR analyses revealed that intracellular Pi levels generally decreased in the Phi-treated *Brassica* sp., and that Phi accumulated in leaves and roots to levels up to 6- and 16-fold that of Pi in Pi-fertilized and Pi-deprived plants, respectively. Moreover, Phi treatment reduced the induction of enzymes (e.g., APase and PPi-dependent phosphofructokinase) and transporters (e.g., high-affinity plasmalemma Pi translocator) characteristic of the Pi starvation response of *Brassica* sp. (6,19). The 75% reduction of APase induction caused by Phi-treatment of Pi-deprived *B. napus* cells was correlated with a similar decrease in the amount of immunoreactive APase protein (19). Increased root:shoot ratio, the hallmark of plant morphological responses to nutrient limitation, was not observed when Pi deprived *B. nigra* seedlings were grown in the presence of 1.5 mM Phi (Fig. 3) (6). Although the precise mechanism whereby Phi exerts these effects is unknown, it was hypothesized that Phi interferes with the signal transduction chain by which *Brassica* sp. detect and respond to Pi deficiency at the molecular level, thereby exacerbating the deleterious effects of Pi starvation (6,19). It should be emphasized that Phi's phytotoxicity was only evident with *Brassica* seedlings and cell cultures that were cultivated under Pi deprived conditions (Fig. 3). The development of Pi fertilized plants was unaffected by the addition of up to 5 mM Phi to the growth media, although they took the anion up from the media and concentrated it within all of their tissues (6). Similarly, hydroponically grown Pi-deprived tomato and pepper plants that were treated with Phi developed P-deficiency symptoms, and also exhibited a significant growth reduction as compared to Pi-fertilized plants (20).

The effect of Pi and Phi on tomato's Pi starvation-induced gene expression was recently analyzed (21). Tomato plants grown in hydroponics were provided with 1 to 3 mM Phi in the presence and absence of 2 mM Pi. Consistent with previous studies (6,19,20), Phi effectively obstructed the morphological and molecular responses normally observed in Pi deprived tomato, and was thus extremely phytotoxic when Pi was absent in the growth media (21). However, Phi's deleterious effects were not obvious in Pi fertilized tomato plants. Expression of Pi starvation inducible genes such as *LePT1* and *LePT2* (high affinity Pi transporters), *LePS2* (APase), and *LePS3* and *TPSI1* (novel genes) was greatly suppressed in Pi starved tomato plants grown in the presence of Phi (21). Immunoblot analyses showed the absence of high affinity plasmalemma Pi transporters in Pi starved tomato roots supplemented with Phi. It was concluded

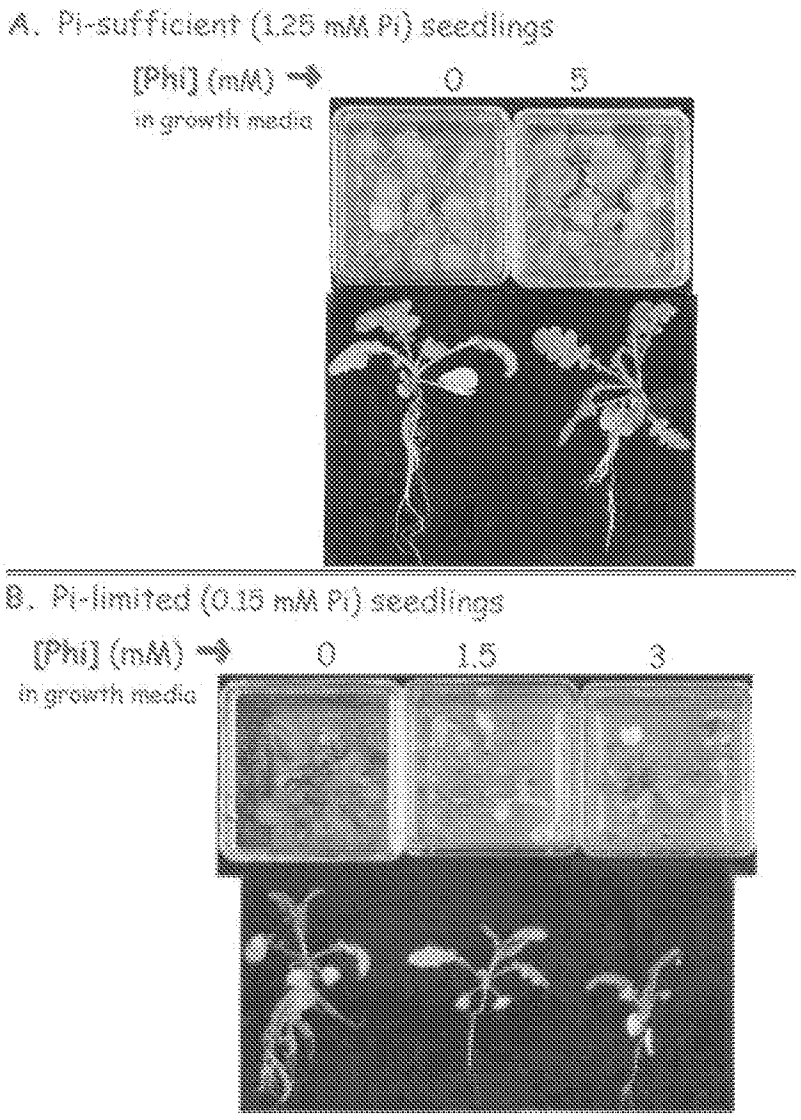


Figure 3. Photograph of Pi-sufficient (top) and Pi-limited (bottom) 20-d-old *B. nigra* (black mustard) seedlings cultivated in the presence and absence of Phi. Surface sterilized *B. nigra* seeds were germinated in plant culture boxes (9 per box) on agar-solidified Murashige-Skoog medium containing 0.7% (w/v) agar and 1.25 mM (**a**) or 0.15 mM K-Pi (**b**) with 0, 1.5, 3, or 5 mM K-Phi as indicated. The plant culture boxes were maintained in a growth cabinet for 20-d at 27°C and a 12:12-h light:dark regime. Adapted from Carswell et al. (6).

that Phi interferes with the normal perception and response mechanism(s) of tomato to Pi deficiency. A similar Phi-mediated suppression of Pi-starvation inducible gene expression was also observed in Pi-deficient tomato cell suspension cultures and *Arabidopsis thaliana* plants [K.G. Ragothama, personal communication].

The impact of Phi on the response of *Saccharomyces cerevisiae* to Pi starvation has also been studied (22). An active Pi-starvation response in this yeast was indicated by a large induction of Pi-repressible APase. When the yeast was cultured in Pi-deficient liquid media containing 0.1 mM Phi, APase derepression and cell development were abolished over the subsequent 48 h culture period. By contrast, treatment with 0.1 mM Phi did not influence the APase activity or growth of Pi-sufficient yeast (22). ^{31}P-NMR spectra obtained from perchloric acid extracts revealed that, as with vascular plants, Phi is assimilated and concentrated to significant levels by yeast cultured with 0.1 mM Phi, especially under conditions of Pi-deprivation. Levels of PPi and poly-P were greatly reduced in the Phi-treated Pi starved yeast cells (22).

In many ways, *S. cerevisiae* appears to respond to Phi in a manner similar to vascular plants. Like plants, *S. cerevisiae* is clearly unable to substitute the P in Phi for the P in Pi. Moreover, Pi deprived plants and yeast that have been treated with Phi suffer far greater deleterious consequences of Pi starvation as compared to those that had not been treated with Phi. Pi-deficient plants and yeast that have assimilated significant amounts of Phi seem to 'sense' that they are Pi sufficient, when in fact their cellular Pi content is very low. Under these conditions Phi prevents plants and yeast from acclimatizing to Pi deprivation by depressing genes encoding enzymes like APase, and high affinity plasmalemma Pi transporters. The results to date support the hypothesis that Phi exerts its effect on the signaling pathway(s) responsible for the detection of, and response to, internal Pi levels. Consistent with this idea was the observation that Phi addition to *B. napus* suspension cells undergoing a transition from Pi sufficiency to Pi deficiency markedly altered the *in vivo* phosphorylation status of several soluble proteins (19). However, Phi had no effect on the *in vitro* activity of endogenous *B. napus* protein kinases, indicating that the anion exerts its effect upstream from the sequence of reactions responsible for differential *in vivo* protein phosphorylation during Pi starvation. The Phi anion would appear to represent a useful tool with which to further investigate the signal transduction pathway by which plants and yeast respond to Pi deprivation at the molecular level.

Is Phosphite a Phosphorus Fertilizer?

Names are important, and so is characterization of the mechanisms by which growth enhancing substances actually work. Call Phi an agricultural

fungicide and in order to register it one must abide by time-consuming and costly regulatory protocols. Call Phi a plant P fertilizer and one can avoid the substantial expenses and tests associated with registering it as a fungicide. Crop producers in many countries are applying formulations containing Phi which are being marketed as a superior source of P nutrition, and yet are intended to supplement regular Pi fertilization programs (23). Despite claims to the contrary (23–26), to our knowledge there is no evidence published in peer-reviewed scientific journals which clearly documents that plants can use Phi as a direct source of P. Phi could, of course, be indirectly providing P to the plant after its oxidation to Pi by soil-dwelling bacteria. However, relative to Pi fertilizers, this is not a cost effective or efficient means of meeting the P requirements of plants (6–8,19–21). It is feasible that some other phenomenon, such as Phi's suppression of plant pathogens, is responsible for the beneficial effects of Phi on plants in field trials. Phi could be effective in reducing low levels of disease, which although asymptomatic, are sufficient to reduce the yield and quality of produce. Certainly the widespread distribution of *Phytophthora* sp. in soil and water and the efficacy of Phi in the control of these plant pathogens make this a possible scenario. Most, if not all, studies represented by the 'Phi=P fertilizer' literature are conducted on plants in the field (23–26). We are unaware of any published results from experiments in laboratories under absolutely controlled conditions. Often the Pi content and microflora of the soil are not taken into account. There is also the issue as to what constitutes an untreated control group. In many studies that are interpreted to indicate that Phi functions as a superior plant P fertilizer, Phi is rarely tested against Pi for its so-called fertilizing ability. Hydroponic and plant cell culture experiments have conclusively demonstrated that Phi is not a P-fertilizer (18–21). If anything, Phi functions as an 'antifertilizer' as it has a profoundly negative influence on plant growth and metabolism when nutritional Pi levels are suboptimal.

Phosphite in the Environment: Concerns and Recommendations

There are several concerns to be addressed regarding Phi's widespread use in agriculture and high-tech industries.[2] Firstly, *Phytophthora* species that are currently sensitive to Phi may become immune to it. This risk is a distinct

[2] Large amounts of hypophosphite ($H_2PO_2^-$; Fig. 1) are being used to reduce metal ions in chemical-plating processes such as those used in compact-disk manufacturing (2). After metal plating, wastewaters containing high concentrations of Phi have been released into the environment. Treatment of Phi-containing industrial wastes is becoming a difficult problem associated with such high-tech industries (2).

possibility and in fact may have already occurred. There has been a report of a naturally occurring Fosetyl-Al resistant isolate of *P. cinnamoni* (9). In this case, plant disease control with Fosetyl-Al was lost after several years of continuous application. At least two Phi-resistant *Phytophthora* strains have been produced by chemical mutagenesis (10). A second potential concern is the effect that repeated Phi treatments of crop plants may have on soil microflora. If significant amounts of Phi accumulate in the soil than there may be a strong selective pressure for microorganisms that are able to utilize Phi as a P source. Conversely, a large influx of Phi into the environment may exert a significant selective pressure against organisms unable to utilize Phi as a source of P. This will undoubtedly influence the microflora of the site, which could consequently have serious repercussions for the other members of the ecosystem. Plants may become vulnerable to Phi by the possible disruption of their symbiotic microbes. For example, roots of the majority of terrestrial plants form symbiotic associations with beneficial mycorrhizal fungi. This improves the ability of the plant to acquire limiting Pi from the environment (5). The results of experiments investigating Phi's effects on mycorrhizal fungi and their associations with roots of vascular plants have been conflicting (27,28). Further investigations into this area would be prudent. An exploration of Phi's influence on the symbiotic relationship between N_2-fixing bacteria (e.g., *Rhizobium*) and leguminous plants should also be conducted.

Although Phi (or Phi containing compounds such as Fosetyl-Al) has been widely employed as agricultural fungicides for several decades (and more recently as 'P fertilizers') recent studies have been a cause for some concern. Phi was traditionally regarded as being metabolically inert in animal and plant systems (9). It is now evident, however, that Phi can evoke marked perturbations in the P metabolism of plants and yeast, and that these effects are very detrimental to their growth under low-Pi conditions (6,19–22). Thus, it is crucial that farmers ensure that their crops are well fertilized with Pi prior to Phi application, or they risk reducing the viability of their crop.

There are regulatory limits to the amounts of Phi which are permitted in food produce. Governmental agencies throughout the world regulate the introduction of potential pest and fungicidal control agents, and set rigorous standards which must be met before any new compound in this class is introduced onto the market. These standards demand that data be presented not only to demonstrate the efficacy of the product, but also that it does not affect human or animal health when present at the levels likely to be encountered in the environment or food products. To meet these standards requires long and rigorous experimentation, which is of necessity, very expensive. These regulations are wise considering the likelihood of significant levels of Phi in products originating from crops previously treated with Phi. One of the features which make Phi such an effective fungicide is that it is retained in the plant for a long time and moves

in the same way as Pi does, often ending up in fruit tissue. Thus, there is an obvious need to document Phi levels in food products derived from Phi-treated crop plants, and to ensure that chronic consumption of these products poses no threat to the public that consume them.

ACKNOWLEDGMENTS

Work in W.C.P's lab is supported by research and equipment grants from the Natural Sciences and Engineering Research Council of Canada (NSERC). We are also grateful to Prof. K.G. Ragothama (Purdue Univ.) and Dr. Julie Niere (Royal Melbourne Institute of Technology) for their helpful discussions and comments on the manuscript.

REFERENCES

1. Adams, F.; Conrad, J.P. Transition of Phosphite to Phosphate in Soils. Soil Sci. **1953**, *75*, 361–371.
2. Ohtake, H.; Wu, H.; Imazu, K.; Anbe, Y.; Kato, J.; Kuroda, A. Bacterial Phosphonate Degradation, Phosphite Oxidation and Polyphosphate Accumulation. Res. Cons. Recy. **1996**, *18*, 25–134.
3. Metcalf, W.W.; Wolfe, R.S. Molecular Genetic Analysis of Phosphite and Hypophosphite Oxidation by *Pseudomonas stutzeri* WM88. J. Bacteriol. **1998**, *180*, 5547–5558.
4. Imazu, K.; Tanaka, S.; Kuroda, A.; Anbe, Y.; Kato, J.; Ohtake, H. Enhanced Utilization of Phosphonate and Phosphite by *Klebsiella aerogenes*. Appl. Environ. Microbiol. **1998**, *64*, 3754–3758.
5. Plaxton, W.C. Metabolic Aspects of Phosphate Starvation in Plants. In *Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes*; Lynch, J.P., Deikman, J., Eds.; American Society of Plant Physiologists: Rockville, MD, 1998; 229–241.
6. Carswell, M.C.; Grant, B.R.; Theodorou, M.E.; Harris, J.; Niere, J.O.; Plaxton, W.D. The Fungicide Phosphonate Disrupts the Phosphate-starvation Response in *Brassica nigra* Seedlings. Plant Physiol. **1996**, *110*, 105–110.
7. Smillie, R.H.; Grant, B.R.; Cribbes, R.L. Determination of Phosphate and Phosphite in Plant Material by Gas Chromatography-mass Spectrometry and Ion Chromatography. J. Chrom. **1988**, *455*, 253–261.
8. MacIntire, W.H.; Winterberg, S.H.; Hardin, L.J.; Sterges, A.J.; Clements, L.B. Fertilizer Evaluation of Certain Phosphorus, Phosphorous, and

Phosphoric Materials by Means of Pot Cultures. Agron. J. **1950**, *42*, 543–549.
9. Guest, D.; Grant, B.R. The Complex Action of Phosphonates as Antifungal Agents. Biol. Rev. **1991**, *66*, 159–187.
10. Fenn, M.E.; Coffey, M.D. Antifungal Activity of Fosetyl-Al and Phosphorous Acid. Phytopathology **1984**, *74*, 606–611.
11. Fenn, M.E.; Coffey, M.D. Quantification of Phosphonate and Ethyl Phosphonate in Tobacco and Tomato Tissues and its Significance for the Mode of Action of Two Phosphonate Fungicides. Phytopathology **1989**, *79*, 76–82.
12. Grant, B.R.; Grant, J.; Harris, J. Inhibition of Growth of *Phytophthora infestans* by Phosphate and Phosphonate in Defined Media. Exp. Mycol. **1992**, *16*, 240–244.
13. Griffith, J.M.; Akins, A.H.; Grant, B.R. Properties of the Phosphate and Phosphite Transport Systems of *Phytophthora palmivora*. Arch. Microbiol. **1992**, *152*, 430–436.
14. Niere, J.O.; Griffith, J.M.; Grant, B.R. ^{31}P-NMR Studies on the Effect of Phosphite on *Phytophthora palmivora*. J. Gen. Microbiol. **1990**, *136*, 147–156.
15. Niere, J.O.; DeAngelis, G.; Grant, B.R. The Effect of Phosphonate on the Acid-soluble Phosphorus Components in the Genus *Phytophthora*. Microbiology **1994**, *140*, 1661–1670.
16. Stehmann, C.; Grant, B.R. Inhibition of Enzymes of the Glycolytic Pathway and Hexose Monophosphate Bypass by Phosphonate. Pest. Biochem. Physiol. **2000**, *67*, 13–24.
17. Smillie, R.; Grant, B.R.; Guest, D. The Mode of Action of Phosphite: Evidence for Both Direct and Indirect Modes of Action on Three *Phytophthora* sp. in Plants. Phytopathology **1989**, *79*, 921–926.
18. Jackson, T.J.; Burgess, T.; Colquhoun, I.; Hardy, G.E.S. Action of the Fungicide Phosphite on *Eucalyptus marginata* Inoculated with *Phytophthora cinnamomi*. Plant Path. **2000**, *49*, 147–154.
19. Carswell, M.C.; Grant, B.R.; Plaxton, W.C. Disruption of the Phosphate-Starvation Response of Oilseed Rape Suspension Cells by the Fungicide Phosphonate. Planta **1997**, *203*, 67–74.
20. Forster, H.; Adaskaveg, J.E.; Kim, D.H.; Stanghellini, M.E. Effect of Phosphite on Tomato and Pepper Plants and on Susceptibility of Pepper to *Phytophthora* Root and Crown Rot in Hydroponic Culture. Plant Path. **1998**, *82*, 1165–1170.
21. Varadarajan, D.; Raghothama, K.G. *Phosphite (HPO_3^{2-}): A Structural Analog of Phosphate ($H_2PO_4^-$) Suppresses Phosphate Starvation Induced Molecular Responses in Tomato*; American Society of Plant Physiologists Annual Meeting: San Diego, CA, 2000; Abiotic Stress #470.

22. McDonald, A.E. 2000. *Suppression of the Phosphate Starvation Response of Saccharomyces cerevisiae by the Fungicide Phosphite*; Queen's University: Kingston, Ontario, Canada, 2000; M.Sc. Thesis.
23. Rickard, D.A. Review of Phosphorus Acid and its Salts as Fertilizer Materials. J. Plant Nutr. **2000**, *23*, 161–180.
24. Lovatt, C.J. A Definitive Test to Determine Whether Phosphite Fertilization Can Replace Phosphate Fertilization to Supply P in the Metabolism of Hass on Duke 7. California Avocado Society Yearbook **1990**, *74*, 61–64.
25. Katz, M. Spoon-feeding Phosphorus. Citrograph **1996**, *81*, 4–6.
26. Lovatt, C.J. Managing Yield with Foliar Fertilization. Citrograph **1998**, *84*, 8–13.
27. Despatie, S.; Furlan, V.; Fortin, J.A. Effects of Successive Applications of Fosetyl-Al on Growth of *Allium cepa* L. Associated with Endomycorrhizal Fungi. Plant and Soil **1989**, *113*, 175–180.
28. Sukarno, N.; Smith, S.E.; Scott, E. The Effect of Fungicides on Vesicular-Arbuscular Mycorrhizal Symbiosis. New Phytol. **1993**, *125*, 139–147.