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Microbial Phytotoxins as Bioherbicide

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Phytotoxins refer to substances produced by plants or other organisms that are toxic to plants. Microorganisms are a lucrative source of phytotoxins. Several species of bacteria, actinomycetes, fungi and algae produce secondary metabolites and by-products of primary metabolic processes which have phytotoxic potential. Several strategies have been laid down for the discovery of these phytotoxic metabolites, which are of agricultural importance. There are different assay formats for the detection of microbial phytotoxins. Many microbial toxins have unique target sites with potential for exploitation by the herbicide industry. These toxins have different modes of phytotoxic action but a few of them shares molecular target sites with some conventional synthetic herbicides. As bio-rational eco-friendly agrochemicals, several host specific and non-host specific microbial phytotoxins are used either directly or as templates to control weed species. The production of microbial phytotoxins is sensitive to a number of diverse factors, which include composition and acidity of media and conditions of culturing etc. Some phytotoxins are isolated from microbes and showed potential bioactivity against various plant/weed species. Some microbial preparations are commercialized such as DeVine®, Collego®, and Dr. BioSedge® etc. while many others are patented. Besides the commercial production of microbial phytotoxins to be used as bioherbicides, different approaches are used to synthesize the analogues of existing bioactive molecules. But, microbial phytotoxins are unstable, have low spectrum of activity and less shelf-life. Despite of these limitations, the microbial phytotoxins may prove to be eco-friendly, non-toxic and selective bioherbicides to be used extensively in the near future as a complementary in integrated weed management systems.

Key words: Phytotoxins, metabolites, bio-rational, bioactivity, bioherbicides, shelf-life.

Agriculture continues to play a major role in Indian economy; contributing approximately 13.9 % of the total national gross domestic production (GDP). In present agriculture, agro-inputs namely water; nutrients and plant protection chemicals are essential inputs to realize higher yields required to feed the burgeoning human population. These inputs need to be managed judiciously for sustainable agriculture, particularly in the Indian context, where a wasteful overexploitation of water has resulted in near scarcity situation and a overuse of synthetic agrochemicals is continuously increasing their toxic levels in food, feed and the environment. The indiscriminate use of synthetic agrochemicals also resulted resistance problem particularly in case of many noxious weed species to synthetic herbicides. Hence, development of innovative strategies for management of the different inputs is imperative to enhance of agricultural productivity and production. But we cannot totally ban the use of synthetic agrochemicals as according to Nobel laureate N. E Borlaug

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"Complete ban on agrochemicals use in agriculture might result in 50% reduction in global food production and 4 to 5 times increase in food prices". Thus we should find a way to combat the problem and one way is by use biopesticides especially microbial phytotoxins.

Phytotoxin refers to a substance produced by a plant that is toxic or a substance that is toxic to the plants. Microorganisms produce a number of toxins as secondary metabolites and these are the by-products of primary physiological processes. Two types of microbial metabolites are there— i) primary metabolires - which are necessary for growth, development and reproduction of organisms and ii) secondary metabolites – these are necessary for protection of organisms against herbivory i.e. it will help in organisms defense system.

The microbial phytotoxins come under secondary metabolites and may be used as bioherbicides in weed management.

Why microbial phytotoxins are used in agriculture?

There are some reasons for using microbial phytotoxins in the field of agriculture-

i. Microorganisms are rich source of biologically active compounds
ii. The field of microorganisms is not well explored in area of agrochemicals but they have potential to be used as bioherbicides.
iii. The compounds are environmentally safe to nontarget organisms
iv. 4.They have unique and novel mode of phytotoxic action
v. The phytotoxins are chemically complex in nature and consists of different stereoisomers imparting less resistance by the weed species.

Sources of microbial secondary metabolites

Phytotoxins are produced by diverse group of microorganisms like prokaryotic bacteria, actinomycetes, fungi and cyanobacteria. Different phytotoxins isolated from microorganism are depicted in (Table 1) (Berdy, 2005).

Chemical class of microbial phytotoxins

The secondary metabolites produced by microbes come under different chemical classes-macro cyclic terpenoides, alkaloids, amino acids, amino sugars, N-heterocycles and O-heterocycles etc.

Steps in discovery of microbial phytotoxins

After purification, very small sample is left and micro bioassay is developed using radical of small-seed plant, small whole plant (duckweed) or leaf disc. After that structure identification and synthesis of analogs are done through quantitative structure activity analysis (QSAR).

Approaches for using microbial phytotoxins

Direct use of fermented products from microorganisms

Different phytotoxins isolated from bacteria, actinomycetes, fungi and cyanobacteria can directly be used for controlling weed species in crop field (Saxena and Pandey, 2001; Duke and Dayan, 2011).

Bacterial Phytotoxins

Several bacterial phytotoxins are used to control plant/weed species. Some of the bacterial phytotoxins are discussed below-

Tabtoxins

It is a dipeptide prophytotoxin produced by *Pseudomonas syringae* pv *tabaci*. It is hydrolyzed *in planta* to form the potent glutamine synthatase (GS) inhibitor tabtoxinine-^{2}-lactam. It is the only host specific phytotoxins for spotted knapweed.

Phaseolotoxin

Phaseolotoxin is a tripeptide produced by *Pseudomonas syringae* pv. *phaseolicola.*

Phaseolotoxin is a protoxin, in that peptidases of the plant must convert it to *N´-(N*1-sulfodiaminophospinhyl)-L-ornithine (PSorn), which is a potent inhibitor of ornithin transcarbamoylase enzyme which is required for arginine synthesis.

Coronatine

It is a jasmonate analog produced by *Pseudomonas coronafacience*. It usurps jasmonate-controlled signaling pathways, thereby deregulating many essential processes. The typical symptom of this toxin is chlorosis of developing tissues and development of hypertrophy.

Actinomycetes phytotoxin

Several actinomycetes phytotoxins are discussed below-

Bialaphos

Streptomyces hygroscopis and *S. viridochromogenes* both produce bialaphos. This tripeptide does not inhibit GS, but must be metabolized in plants and microbes to L-phosphinothricin, the active GS inhibitor. Inhibition

of GS causes accumulation of toxic levels of ammonium, as well as a disruption of amino acid and other primary metabolism. One of the earliest general physiological effects is cessation of photosynthesis. Both bialaphos and phosphinothricin are sold as commercial herbicide (glufosinate).

Oxetin

It is produced by *Streptomyces* sp. and is a weak GS inhibitor.

Phosalacine

It is produced by *Kitasatosporia phosalacinea*, and it releases phosphinothricin upon hydrolysis in plant and inhibit GS.

Hydantocidin

Hydantocidin is a spironucleoside from *Streptomyces hygroscopis*, is highly phytotoxic. It is phosphorylated *in vivo*, and the derivative, 5'-phosphohydantocidin (5PH), inhibits adenylosuccinate synthetase, an enzyme required for purine synthesis.

Actinonin

Actinonin, a product of an *Actinomyces* MG848-hF6, inhibits plastid peptide deformylase, an enzyme required for *N*-terminal protein processing of plastid-encoded proteins. This compound is a non-selective herbicide that results in chlorotic plants.

Phthoxazolin

It is isolated from *Streptomyces* sp. and inhibits cellulose biosynthesis in plant.

Pyridazocidin

A cationic compound from *Streptomyces* sp. causes rapid plant necrosis and chlorosis, like paraquat. Its mode of action is exactly like bipyridiniums, diverting electrons from photosystem I to become reduced to a reactive radicle that subsequently generates superoxide radicle, resulting in a cascade of destructive oxidative processes.

Nigericin

It is a product of *Streptomyces hygroscopicus*, is an uncoupler of photophosphorylation. It inhibits photosynthesis with decreased ATP/ADP ratios, decreased energy quenching, and hyper-reduction of QA.

Some other examples are Resormycin,

Table 1. Number of secondary metabolites so far isolated from microorganisms

Microorganisms	No. of metabolites isolated
Prokaryotic bacteria	3800 (17%)
Actinomycetes	10000 (45%)
Streptomyces sp.	7500
Rare actinomycetes	2500
Fungi	8600 (38%)
Protozoa	50
Algae	1276
Marine microbes	7323

Table 2. Herbicidal activities of fatty acid and structural analogues on *Lemna minor*

Acid	MIC (µg/plant)
Decanoic acid	30-60
2,4-Decadienoic acid	30
2,4,7-Decatrienoic acid	30
1-Decanol	>60
2-Decanone	>60

Table 3. Toxicity of drazepinone in leaf puncture assay

Plant/weed	Toxicity
Duram wheat	Necrosis, diameter 2-3 mm
Chenopodium album	Necrosis, 3-5 mm dia
Perennial ryegrass	Necrosis, 1-2 mm dia
Amaranthus retroflexus	No symptoms
Sonchus arvensis L	Necrosis, 2-3 mm dia
Bromus sp.	No symptoms

Table 4. Microbial phytotoxins patented for use as herbicides

Chemical class	Example
Amino acid	Rhizobiotoxin
	Gabaculine
	Homoalasonone
Phosphinates	Phosphinothricine
	Bialaphos
	Phosalacine
Miscellaneous	Oxetin
	Irpexil
	Moniliformin
	Herbicidins
Nucleosides	Tubericidin
	Formycin A
Glutarimides	Cycloheximides
	Streptimidone
Macrocycles	Herbimycins
	Ascotoxins
Sugars	Nojirimycin

vulgamycin, anisomycin, herbimycin, herboxidiene, toyocamycine, homoalanosine etc.

Fungal phytotoxins

They are divided into host-specific and non host-specific
phytotoxins

Host-specific phytotoxins: Some host-specific phytotoxins are-

Maculosin

It is a cyclic dipeptide, produced by *Alternaria alternata* and is the host specific phytotoxin to spotted knapweed.

Bipolaroxin

It is produced by Bipolaris cyanodontis, a fungal pathogen of Bermuda grass. Bipolaroxin causes phytotoxicity to wild oats, sugarcane and maize.

AAL-toxin

AAL toxins are produced by *Alternaria alternate* f. sp. *lycopersici.* It cause wilting and necrosis and light dependent plasma membrane & tonoplast disruption.

Non host-specific phytotoxins: Several non host-specific phytotoxins are reported and these are –

Conexistin

It is produced by *Paecilomyces variotii* SANK 21086. It inhibits aspertate aminotransferase. It is ctive against both monocot and dicot plants, with selective protection to corn.

Tentoxin

It is a cyclic tetrapeptide from the plant pathogen *Alternaria alternata*, inhibits chloroplast development, which phenotypically manifests itself as chlorotic tissue. It inhibit energy transfer of the chloroplast-localized CF1 ATPase.

Zinniol

Zinniol, a product of several *Alternaria* species and one *Phoma macdonaldii*, binds plant protoplasts and stimulates Ca^{++} entry into cells. It may act on a specific class of plant calcium channel.

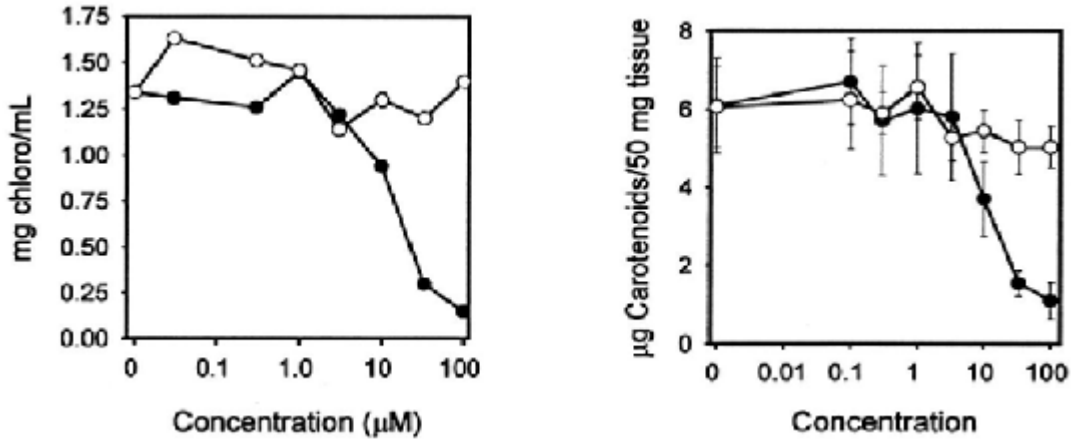

Fig. 1. Effect of usnic acid on chlorophyll conc. lettuce cotyledon after 6 days of growth

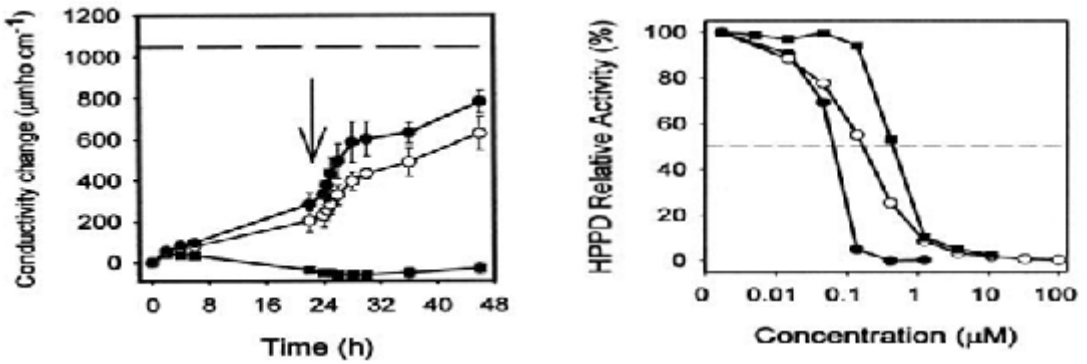

Fig. 2. Effect of usnic acid on electrolyte leakage from cucumber cotyledons

Prehelminthosporl

It is produced by Helminthosporium sp. It inhibits plasma membrane ATPase activity.

Victorin

Victorin, a fungal product of *Cochiobolus victoriae*, induces a collapse of the mitochondrial transmembrane potential, which results in a mitochondrial membrane transition. It also binds the P protein of the glycine decarboxylase complex of the mitochrondria. All of this has been associated with programmed cell death.

Hymeglusin

It is a phytotoxin produced by *Fusarium* sp. It inhibits 3-hydroxy-3-methylglutaryl coenzyme A synthase of plants and animals. This enzyme is required for synthesis of certain terpenoids (e.g., sterols) of the mevalonic acid pathway in plants.

Colletotrichin

Colletotrichin is a highly phytotoxic compound from several *Colletotrichum* species. Ultrastructurally, the first effect of this compound is disintegration of the plasma membrane, accompanied by massive cellular leakage. The effect is not light dependent and could not be reversed with antioxidants, suggesting that it has a direct effect on the plasma membrane.

Cercosporin

Cercosporin, is a red fungal toxin from *Cercospora* sp.This photodynamic pigment is a potent photosensitizer and, in the presence of light and oxygen, it generates singlet oxygen ($^1O_2$) and superoxide (O^{2-}) ions that induce rapid membrane peroxidation and cellular death.

Ophiobolin

Ophiobolins, tricyclic sesquiterpene

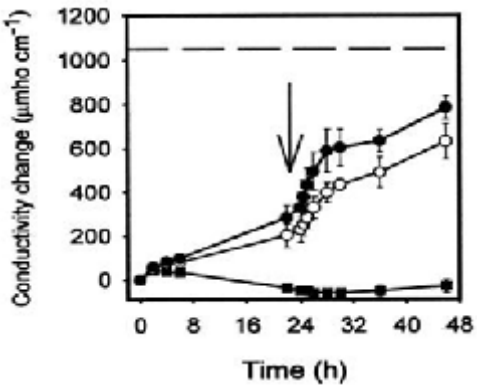

Fig. 3. Effect of usnic acid on activity of HPPD

phytotoxins from certain species of *Bipolaris*, cause many symptoms on plants that were considered to be largely due to effects on the plasma membrane. It effects on maize root ion leakage correlate well with its direct antagonism of calmodulin. Its effects on calmodulin cause inhibition of transport of nuclear-coded proteins into both the mitochondrion and the plastid.

Phomalactone and nigrosporin

Both are produced by *Nigrospora* sp. and cause rapid electrolytic leakage of host tissue.

Phytotoxins from Algae

Some algal originated phytotoxins (fig.1) are-

Cyanobacterin

Cyanobacterin is a halogenated compound from the freshwater cyanobacterium *Scytonema hofmanni* that inhibits electron transport of photosystem II.

Fischellin A

Fischerellin from the cyanobacterium *Fischerella muscicola* produces fischerellin A that inhibits PSII of green algae and higher plants. Besides some terpenoides and phenolic phytotoxins were also isolated from algae.

Synthesizing the analogues of existing bioactive molecules

The new synthetic analogs can be prepared using the phytotoxins as lead molecules. Methoxyphenone, a synthetic analog of anisomycin is used extensively in rice to control weed.

Factors affecting *in vitro* formation of microbial phytotoxins

In order to isolate phytotoxins (PTs) in amounts sufficient for studies of chemical and biological properties, the fungi are cultured in liquid nutrient media (Berestetskiy, 2008). The factors are-

Composition of nutrient media

The yield of PTs may be increased by selecting appropriate carbon and nitrogen sources and finding the right combination (in terms of the concentrations and the molar ratio). Certain trace elements may affect considerably toxin production by fungi. For this reason, it is recommended to use artificial media (rich in vitamins and trace elements). For example, Strains of *Fusarium oxysporum* form fusaric acid only in the presence of Zn ions (0.08–0.4 g/l)

Medium acidity

During culturing, the concentration of

hydrogen ions in the medium may affect the activity of fungal enzymes and the stability of secondary metabolites. The formation of aspergillomarasmin A by *Pyrenophora teres* grown in modified de Vries medium was augmented considerably upon its neutralization.

Water activity and medium tonicity

Under stress conditions, however, e.g., when moisture content is insufficient, toxin synthesis is frequently increased. Thus, certain strains of *Alternaria alternate* and *Phoma* sp., produce, respectively, tenuazonic acid and the antibiotic squalestatin, when water activity is low.

Toxin formation stimulants

Certain plant metabolites stimulate the formation of PTs and potentiate their activity. Germination of conidia of *Alternaria brassicicola* is associated with the release of compounds that stimulate the synthesis of oligosaccharides in the leaves of alternariosis-susceptible cabbage cultivars, which in turn stimulate the production of the pathotoxin by the fungus.

Conditions of culturing

The formation of certain PTs (such as cercosporin) requires light.

Toxin formation by fungi may be stimulated under the conditions of cold stress. Thus, a strain of *Fusarium sporotrichiella* actively forms T-2 mycotoxin, if the fungus is cultured on millet for one week, first at 18°C and then at 5°C.

Duration of culturing

Some PTs are detected in the medium early on, in a matter of days or hours after the germination of the conidia. Maximum accumulation of the PT of *Bipolaris euphorbiae* in the culture liquid was observed after six or seven days, coinciding with the peak of biomass gain, minimum pH (4.2) and near-complete consumption of the carbon source.

Mechanisms of microbial phytotoxins resistance by plants/weeds

Plants developed some measures (Berestetskiy, 2008) by which they show resistant to phytotoxins. The mechanisms are-

Secretion of plant metabolites

When acted upon by plant metabolites, PTs may undergo structural alterations changing their activity. Thus, solanapyrone A (a PT from *Ascochyta rabiei*) forms a less toxic complex with glutathione in chickpea cells. The higher the activity of glutathione S-transferase, the greater is the resistance of these cells to the toxin.

Chemical alteration of phytotoxins

Plants resistant to *Alternaria brassicae* (e.g., *Sinapis alba*) are capable of degrading the toxin it produces (destruxin B). The degradation process involves by hydroxylation followed by glucosylation.

Changing structure and conformations of target sites

Plant cells are insensitive to PTs in the absence of appropriate molecular targets. It is possible to block the effects of a PT by changing the structure and/or conformation of its targets. Such approaches would affect the operation of plant genes (expression, introduction of mutations, etc.) associated with the synthesis of the PT target. For mexample, maize cultivars devoid of the gene URF13 are insensitive to T-toxin.

Detection of microbial phytotoxins

Brief descriptions of assay used in detection of microbial phytotoxins at preliminary screening level (Stonard and Miller-Wideman, 1994) are-

Whole plant, excised leaf, and algal assay

Here after applying test solution to whole plant or adaxial part of leaf, the visual symptoms are recorded like growth inhibition, root elongation, necrotic spot on leaf etc. Herbimycin, homoalanosine; colletotrichin, AM-toxin are detected by whole plant and excised leaf assay repectively.

Seed germination inhibition

After applying test solution of hydantocidin, viridiol etc. to the seed kept upon moistened filter paper, the set up is incubated. The germination inhibition is rated by observing visual growth.

Wheat coleoptile assay

Prehelminthosporol, cytochalasins, and ophiobolin are assayed by using wheat coleoptiles maintained in phosphate buffer supplemented in 2% sucrose solution. After certain time the coleoptile length is measured.

Callus and cell suspension assay

Plant growth inhibitory activity of compactin and zinniol was demonstrated using tobacco callus added to appropriate medium containing different concentrations of test material. After 30 days fresh weight of callus was measured.

Photosynthesis inhibition assay

Here pumpkin cotyledon leaf discs are floated on a phosphate based medium under light. In presence of photosynthesis inhibitor like 5-methylthio-1.2-dithiolane, the leaf discs become oxygen depleted, lose buoyancy, and sink.

Antimetabolite and enzyme inhibitor assay

Oxetin and phosalacine are assayed using this method.

Translocation assay

Test solution of phosidomycin is applied to castor bean cotyledons and exudates are collected from base of cotyledon and analyzed for the presence of test compound. This assay determines the phloem transport capabilities of phytotoxin.

Case studies

Two Herbicidal Metabolites from *Streptomyces viridochromogenes* tu 6105

(2E, 4Z)-Decadienoic acid and (2E, 4Z, 7Z)-Decatrienoic acid were isolated from above mentioned *Steptomyces* sp. The compounds were purified by column chromatography followed by HPLC. The biological activity of the compounds was done on two weed species- *Lemna minor* and *Lepidium sativum*. The visual symptoms i.e. inhibition of growth, bleaching and inhition of germinations were recorded. The minimal inhibition concentration (MIC) was calculated (Table 2) which the lowest conc. is causing growth inhibition (Armin *et al*., 1999). Both the compounds showed highest and equal toxicity to *Lemna minor*. The toxicity decreases if acid is replaced by alcohol or aldehyde.

Herbicidal activity of drazepinone produced by *Drechslera siccans*

Drazepinone was isolated from fungus using chloroform and purification was done by column chromatography using chloroform-iso propanol. Further purification was done by silica gel chromatography using ethyl acetate. The compound was applied on leaf at conc. of 2µg/µL and the leaves were kept at 25^0C temperature under fluorescent light for development of visual symptoms (Table 3). All the plant/weed species developed necrotic symptoms except *Amaranthus* and broom grass (Evidente *et al*., 2005).

Phytotoxic lichen metabolite, potent inhibitor of plant *p*-hydroxyphenylpyruvate dioxygenase (HPPD)

(-)-Usnic acid was isolated from Alectoria sarmentosa by acetone extraction followed by sonication with dicloromethane. Recrystallization of the compound was done using ether and chloroform-methanol. The compound was applied to lettuce seed, cucumber cotyledon to see the dose response assay and effect of (-) and (+) usnic acid along with commercial herbicide sulcotrione on chlorophyll & carotenoid content, membrane integrity and HPPD activity (Romagni *et al*., 2000). (-) usnic acid inhibits both chlorophyll and carotenoid synthesis (Fig. 1). (-) usnic acid also cause light dependent electrolyte leakage on cucumber cotyledon (Fig. 2). The compound was also found to be better inhibitor of HPPD than commercial herbicide sulcotrione (Fig. 3).

Commercial bioherbicides

Some microbial preparations are commercialised (Li *et al*., 2003) to be used as bioherbicides in Canada, USA, China, and Japan. Many microbial phytotoxins are patented in different states (Table 4). Some commercialised products are-

i) Lubao®: It is based on *Colletotrichum gloeosporioides f. sp. cuscutae* formulation, developed in China 1963 for controlling Cuscata sp. in soybean.

ii) DeVine®: It is based on *Phytophthora palmivora* and first product to be fully registered as amycoherbicide in 1981. It kills strangler wine – a problematic weed in citrus plantation of Florida.

iii) Collego®: It is based on *Colletotrichum gloeosporioides f. sp. aeschynemone*. It was used to control joint vetch in rice and soybean.

iv) BioMal ®: It contains spores of *Colletotrichum gleosporiodes f. sp. malvae.* It was used to control *Malva pusilla* in Canada and USA.

v) Dr. BioSedge®: It was based on the rust fungus *Puccinia canalicuta* and used to control *Cyprus esculantus* L. in soybeans, sugarcane, maize, potato & cotton but was failed due to uneconomic production system & resistance in some weed biotypes.

vi) Stumpout™: It is based on *Cylindrobasidium leave* and was used to control *Acacia* species in native vegetation.

Limitations of microbial phytotoxins as bioherbicide

Besides many advantages of

bioherbicides, certain factors have been reported to limit the development of bioherbicides into commercial products. These include biological constraints (host variability, host range resistance mechanisms and interaction with other microorganisms that affect efficacy), environment constraints (epidemiology of bioherbicides dependent on optimum environmental conditions), and technical constraints (mass production and formulations development of reliable and efficacious bioherbicide), and commercial limitations (market size, patent protection, secrecy and regulations) (Charudattan & Dinoor, 2000).

Future prospects of microbial phytotoxins

The bioherbicides approach is gaining momentum. New bioherbicides will find place in irrigated lands, wastelands as well as in mimic parasite weeds or resistant weed control. Using simple lead molecules and with the knowledge of chemoinfirmatics we can maximised the use of phytotoxin.

CONCLUSION

Microbes contain a virtually untapped reservoir of phytotoxins having potential to control weeds and can be used directly or as templates for synthetic herbicides. Though the lead molecules are unstable and chemically complex but still it has enough scope to be used in integrated weed management systems. The present over-reliance on chemical herbicides and the tendency to base weed-management decisions purely on economic considerations, at the exclusion of ecological and societal benefits, is a serious limitation that could stifle biological control.

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