# UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería, Ciencias y Administración

Doctorado en Ciencias de Recursos Naturales



# MICROBIAL DEGRADATION OF IPRODIONE AND ITS METABOLITE 3,5-DICHLOROANILINE (3,5-DCA) BY A DEGRADING BACTERIAL INOCULUM AND ITS APPLICATION IN THE GRASS COVER OF A BIOBED

**DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES**


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**Microbial degradation of iprodione and its metabolite 3,5-dichloroaniline (3,5-DCA) by a degrading bacterial inoculum and its application in the grass cover of a biobed**

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***"En honor a quienes desinteresadamente aportaron a la realización de esta tesis"***

***En especial a mi madre, por instruirme en lo esencial de la vida y a mi novia por ayudarme a edificar los cimiento de nuestro futuro***

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## Summary and outline of this thesis

In Chile, one of the most sold pesticides is dicarboxiamide fungicide iprodione. When iprodione is applied, it mainly reaches the soil where adapted microorganism such as degrading bacteria are able to hydrolyze iprodione into its main 3,5-dichloroaniline (3,5-DCA) metabolite through formation of 3,5-dichlorophenyl-carboxamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II). However, due to iprodione and 3,5-DCA are deleterious molecules, their accumulation in the environment should to be mitigated. In this context, an interesting alternative might be given by the relationship between plant roots and degrading bacteria in a process called rhizodegradation. In this context, it is expected that rhizodegradation occurs strongly in places with a large exposition to pesticides such as the grass cover of biobed. Biobeds are biopurification systems (BPS) constituted by a biomixture generally composed by soil, peat and straw, which is covered by a grass layer offering a suitable pesticide mitigation, possibly by an active rhizodegradation. Nevertheless, it is possible that rhizodegradation failure due to several factors, being in these cases the inoculation of grass cover with characterized degrading bacteria a suitable strategy to improve the process. Therefore, the main objective of this thesis considered to evaluate the microbial degradation of iprodione and its metabolite 3,5-DCA by a degrading bacterial inoculum and their application in the grass cover of a biobed. Here, laboratory enrichments achieved the isolation of a partial 3,5-DCA-degrading *Pseudomonas* sp. strain and an iprodione-degrading consortium composed by an *Arthrobacter* sp. strain C1 and an *Achromobacter* sp. strain C2 from an acidic pristine soil. Posterior studies revealed that *Arthrobacter* sp. strain C1 was the key iprodione-degrading strain, which was able to maintain its degrading capacity in a wide range of temperature and pH and in the co-presence of other pesticides. Also was demonstrated that iprodione catabolism was done by strain C1, whereas strain C2 only could co-metabolize it. Additionally, strains showed only difficulties to catabolize metabolite I, whereas no problems to co-metabolize metabolites I and II were observed, suggesting that an enough biomass was necessary to transform metabolite I, possibly through a detoxification process. Furthermore, strain C1 demonstrated ability to degrade vinclozolin and propanil, constituting the first evidence about cross degradation among these pesticides. Afterward, antagonistic effect of indigenous soil microorganisms on the development of our strains and tolerance of *Lolium perenne* (ryegrass) seeds to germinate

in presence of high iprodione and 3,5-DCA concentration demonstrated that bacteria as well as ryegrass seeds were not noticeably affected, proposing them as suitable subjects to be tested in later rhizodegrading experiments. However, the limited metabolic ability of *Pseudomonas* sp. to degrade 3,5-DCA discarded its utilization in posterior steps. Rhizodegrading experiments demonstrated that iprodione removal rates were improved notoriously by inoculation of strain C1 in biomixture, rates that were enhanced when bacterial inoculum was incorporated close to the rhizosphere of ryegrass. Therefore, increasing biological activities revealed that microorganisms and roots had active roles in the removal of iprodione. Nonetheless, changes for fungi and bacterial abundances did not show clear patterns for bacterial inoculation and root presence, revealing that removal was not determined by the microbial abundances. Thus, this study demonstrated that *Arthrobacter* sp. strain C1 was an effective iprodione-degrading subject, whose incorporation into BPS might be a suitable strategy to enhance the removal of iprodione. Future perspectives for the mechanisms involved in the iprodione metabolic pathway and the utilization of *Arthrobacter* sp. strain C1 as inoculum are discussed.

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# Chapter 1

## General Introduction

# General Introduction

## 1.1. Introduction

Currently, the global demand of pesticides have seen a steady increase every year in order to satisfy the constant need of these agrochemicals from the food production industry. In this context, Chile has been cataloged by the Organization for Economic Co-operation and Development (OECD) as one of its member countries with the strongest agricultures, characterized by an increasing production and an expanding land area designated to the activity (OECD, 2013) As result, Chilean agriculture has been ranked among those with the highest application of pesticides per $ha^2$ of arable land (Stokstad and Grullon, 2013), phenomenon that is a possible reflex of the highest records of sold pesticides during recent years (SAG, 2008, 2010). In this sense, just the fungicide/bactericide series has represented more than 50% of total pesticides sold, being iprodione [3-(3,5-dichlorophenyl)N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] one of the most used chemical fungicides for this period (SAG, 2010).

Iprodione is an effective contact fungicide recommended to control fungal pests such as *Botrytis cinerea*, *Alternaria* sp. *Cladosporium* sp., *Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., *Penicillium* sp. and *Sclerotinia* sp., affecting diverse crops such as strawberry, tobacco, pear, lettuce, onion, vineyard and ornamentals plants (AFIPA; Miñambres *et al*., 2009; Grabke *et al*., 2014). This fungicide acts cutting off the signal transduction in glycerol synthesis, consequently affecting the normal hyphal development and spore germination of a broad spectrum of fungi (Ochiai *et al*., 2002). However, it has been recently proven that iprodione can work as a potent controlling agent of harmful root parasitic soil nematodes such as the cyst-forming genera *Heterodera* or *Globodera* and/or the root knot-forming genus *Meloidogyme*, which constitute a novel outlook for the protection of crops (Leysen and De Kerpel, 2012).

Iprodione is moderately toxic to several little animals and for humans it is considered probably carcinogenic (USEPA, 1998). These adverse effects have been indicated as result of oxidative damage by iprodione on cells through the production of free oxygen radicals and its bind to the aryl hydrocarbon receptor (AhR) able to induce the cytochrome P450 system *in vitro* (Radice *et al*., 2001; Ferraris *et al*., 2005). Iprodione is quite insoluble in water (12.2 mg $L^{-1}$) and its $K_{oc}$ of 400 mL $g^{-1}$ denotes a not strong adsorption into the soil particles (Strömqvist and Jarvis, 2005). Additionally, its moderate

groundwater ubiquitous score (GUS) of 2.02 x $10^{-1}$ µg $L^{-1}$ (FOOTPRINT, 2015) confers it a relative mobility to contaminate groundwaters or run-off into surface waters (Derbalah *et al*., 2003; Sequinatto *et al*., 2013). Nonetheless, due to the presence of the amino and aromatic group in the structure of iprodione and metabolites, these molecules are subject to be coupled by means of covalent bounds (Kong *et al*., 2013) to the humic acids present in soil colloids resulting in a relatively strong adsorptions in soils with higher soil organic matter (SOM) content (Adrian *et al*., 1989a; 1989b; Morales *et al*., 2013). In addition, it has been demonstrated that iprodione is prone to be transformed into several isomers in neutral to alkaline systems such as water and soil, being the parent molecule not transformed into breakdown products but structurally rearranged (Athiel *et al*., 1995; Zadra *et al*., 2006). On the other hand, it has been demonstrated that iprodione is more persistent and stable in acidic soils (Cayley and Hide, 1980; Walker, 1987). However, enhanced iprodione removal has been well documented for soil with previous and repeated applications of the fungicide, independent from the soil chemical nature (Walker, 1987; Martin *et al*., 1990). In these cases, this accelerating removal has been indicated as the result of a microbial degradation by adapted microorganisms, which constitute the main controlling way of iprodione and other dicarboxiamide fungicides in soils (García-Cazorla and Xirau-Vayreda, 2005). Although to the biotic iprodione removal is proven, scarce information about the effect of this fungicide on soil microbial communities has been reported until now. Therefore, it has been documented that fungi populations are adversely affected whereas bacteria are able to increase their populations or not modified them as response to the iprodione contamination, conferring them the main responsible of iprodione attenuation in soils (Wang *et al*., 2004; Miñambres *et al*., 2009).

Soils showing enhanced removal of iprodione have been used to isolate degrading bacterial strains involved in the metabolism of the fungicide and its metabolites. Therefore, studies carried out by Athiel *et al*., (1995) led to the first isolation of an *Arthrobacter*-like strain bacteria able to rapidly degrade iprodione. However, scarce understanding about the metabolism of this strain could be investigated by these researchers. In a posterior work, Mercadier *et al*., (1997) noticed the isolation of some *Pseudomonas* strains able to synergistically metabolize iprodione via formation of three intermediate metabolites. Thereby, a first amydohydrolysis was able to produce isopropylamine and 3,5-dichlorophenyl-carboxamide (metabolite I), followed by a

second hydrolysis to transform metabolite I into 3,5-dichlorophenylurea-acetate (metabolite II) and a final amydohydrolysis to release 3,5-dichloroaniline (3,5-DCA), constituting the main iprodione metabolic pathway reported until now (**Figure 1.1**).

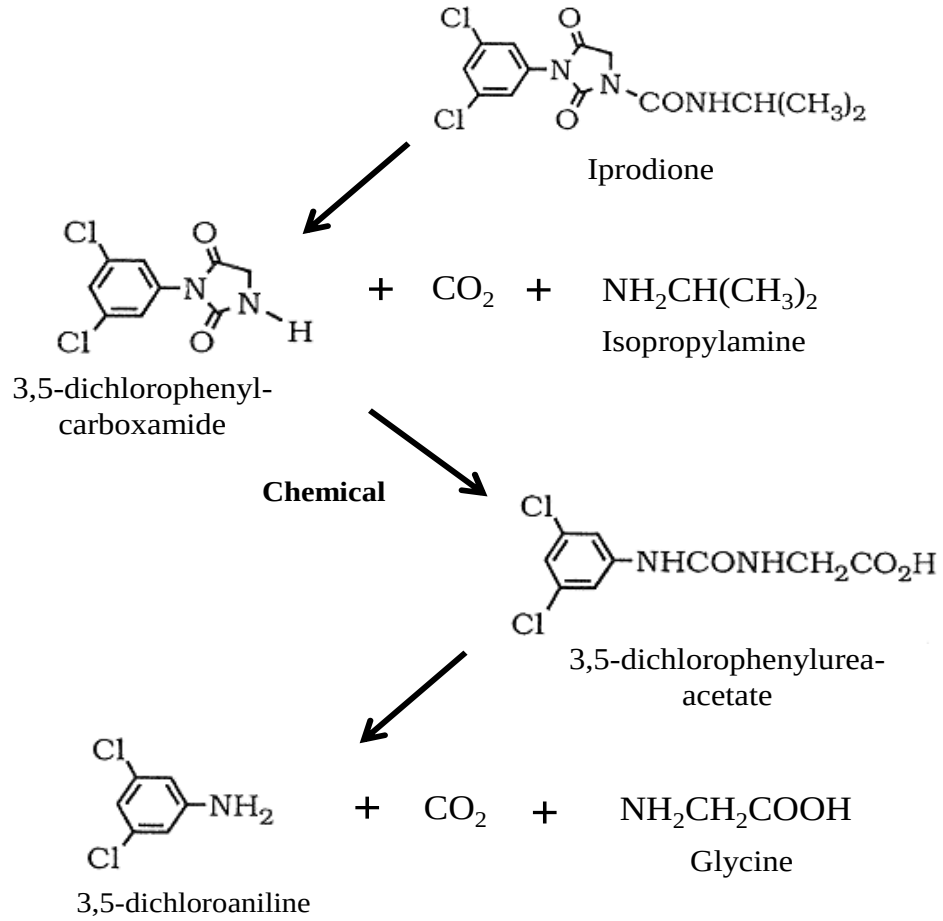


**Figure 1.1.** Iprodione metabolic pathway according to Mercadier *et al.*, (1997). The arrows represents each molecular transformation accompained by the release of intermediate metabolites and subproducts when this correspond.

This 3,5-DCA metabolite is quite persistent in soil (PBT Profiler) and has been reported as a potent nephrotoxic for rats (Lo *et al*., 1990) and toxic for soil microorganisms (Rochkind *et al*., 1986). This molecule is rather soluble in water (784 mg $L^{-1}$) and moderately mobile in soils according to its $K_{oc}$ of 310 mL $g^{-1}$ (Sabljić *et al*., 1995). However, as 3,5-DCA is composed by an high reactive aromatic amino group able to bind strongly to humus or SOM, it is expected that its mobility may be much lower in organic soils (Adrian *et al*., 1989b; Dec and Bollag, 1988). As it was mentioned above, 3,5-DCA is considered the most reactive and toxic of the dichloroaniline isomers, being its bulky

chlorine halogenations able to exert a high van de Waals radius (Valentovic *et al*., 1995; Padmanabhan *et al*., 2006). As result, 3,5-DCA has been cataloged as a rather stable molecule to be metabolized by microorganism, fact that might explain the scarce amount of studies reporting the isolation of effective 3,5-DCA-degrading bacterial strains (Travkin and Golovleva, 2003; Lee *et al*., 2008; Hongsawat and Vangnai, 2011; Yao *et al*., 2011). Considering all the above mentions, soil degradation of iprodione could be an important environment concern due to the lesser probability to mineralize its 3,5-DCA metabolite, which might be deleterious for human health and environmental quality and should be mitigated.

Potential of isolated bacterial strains able to metabolize contaminants as iprodione and/or 3,5-DCA has been well documented. However, the applicability of these strains might need extra-technologies to ensure an effective bioremedial process (Boopathy, 2000). In this context, an interesting alternative might be given by the close relationship between plant roots and rhizospheric bacteria, also known as rhizobacteria. In it, plants can interact closely with beneficial bacteria in removal of pesticides by releasing root exudates in a process called rhizodegradation (Gerhardt *et al*., 2009). This interaction is able to increase microbial diversity in root zone where physical, chemical and biological parameters are modified in presence of root exudates (Dennis *et al*., 2010). Density and diversity of rhizospheric bacteria have been demonstrated to be at least twice higher than bulk soil (non-rhizosphere) with increment in numbers of biological activity in degradation of pesticide (Zelenev *et al*., 2005). Therefore, plants are able to recruit beneficial bacteria to root surface through releasing some short-chain organic acids such as oxalate, malate and citrate inside the root exudates (Tan *et al*., 2013). On the other hand, it is possible that organic acid exudation could facilitate rhizodegradation by chelation of polyvalent cations improving the bioavailability of organic contaminants by dissolution of SOM complexes, where these molecules are retained (Yang *et al*., 2001; Ou *et al*., 2015). Accordingly, rhizodegradation is a multifactorial process, where plants play a relevant role. However, presence of bacterial populations with the competent metabolism to mineralize pesticides seems to be the key aspect for effective removal (Gerhardt *et al*., 2009).

As it was explained above, the repeated exposition to high levels of pesticides constitute the main way by which microorganisms gain extra metabolic abilities to degrade target pesticides (Arbeli and Fuentes, 2007). Therefore, it is expected that

rhizodegradation occurs strongly in places with a large exposition at high level of pesticides as on-farm biobed systems. Biobeds are biopurification systems (BPS) originated as a simple, low-cost and effective alternative to minimize environmental contamination from pesticide during its manipulation, especially when filling the spraying equipment on-farm (Castillo *et al*., 2008). They are generally composed by a mixture of top soil, peat and straw and covered with a grass cover. Biomixture component offers an adequate pesticide sorption and effective degradation due to indigenous microorganisms present in soil. Straw stimulates extracellular ligninolytic enzyme production by microorganisms. Peat gives a potential sorption capacity, adequate moisture and a low pH adequate to enzyme activity (Castillo and Torstensson, 2007). Finally, grass cover helps keep the biomixture humidity, reveals pesticide spills due to point damage in grass (Torstensson and Castillo, 1997) and the most important feature is that it could improve the pesticide removal for the retained pesticides in the first biobed layer by an active rhizodegrading process (Fogg *et al*., 2004b). It is known that increasing the activity of indigenous pesticide-degrading microorganisms contained in the soil components of biomixture, is the key factor for a suitable BPS performance (Castillo *et al*., 2008). Thereby, to reach optima removal rates, biomixtures should ensure previously a suitable proliferation of its microorganisms (Sniegowski *et al*., 2011), aspect that is not often possible to achieve as result of adverse environmental conditions, pesticide nature and inadequate metabolic ability of microorganisms (Fogg *et al*., 2004a; Gerhardt *et al*., 2009). In these kind of situations, inoculation of biomixture with characterized and effective pesticide-degrading microorganisms seems to be a suitable strategy to improve the removal, avoiding the accumulation of pesticide and their metabolites (El Fantroussi and Agathos, 2005). Therefore, several studies reporting the inoculation of biomixture with white-rot fungi (von Wirén-Lehr *et al*., 2001; Bending *et al*., 2002; Ruiz-Hidalgo *et al*., 2014) and application of primed materials containing degrading microorganisms, such as soils with large history of exposure to pesticide (Sniegowski *et al*., 2011, 2012), have been subjects of investigation. Nonetheless, studies evaluating pesticide removal by incorporation of pesticide-degrading bacteria in BPS have been scarcely reported (Verhagen *et al*., 2013), being null the approaches related to the rhizodegradation improvement. Therefore, to enhance rapid and complete pesticide rhizodegradation by inoculation of the BPS grass cover with specialized pesticide-degrading bacterial isolates can be considered. However, a deep acknowledgment about inherent characteristic of bacterial inoculum such as degrading mechanisms (metabolism or co-metabolism) (Nzila,

2013), metabolic pathways (Heinaru *et al*., 2005), degrading versatility and growth rates (van Veen *et al*., 1997) are fundamental aspects to consider for the selection of the best subjects. Finally, biotic and abiotic affecting factors such as competition with indigenous microorganisms for the carbon sources, antagonistic interaction, predation, temperature, moisture, pH, organic matter content and nutrient availability (Mrozik and Piotrowska-Seget, 2010; Ray, 2014), are also important variants to evaluate previously in order to use a determinate bacterial inoculum as bioremedial subjects in prospect.

## 1.2. Hypothesis and research objectives

### 1.2.1. Hypothesis

Inoculation of iprodione- and 3,5-DCA-degrading bacteria isolated from rhizosphere soil will be able to improve their removal rates in the grass cover of a biobed.

### 1.2.2. Research objectives

#### 1.2.2.1. General objective

To evaluate the microbial degradation of iprodione and its metabolite 3,5-DCA by a degrading bacterial inoculum and its application in the grass cover of a biobed.

#### 1.2.2.2. Specific objectives

I. To isolate and characterize iprodione- and 3,5-DCA-degrading bacteria from soil with and without historical pesticide applications.

II. To elucidate the metabolic pathway of iprodione by soil bacterial isolates and identify their role in the degradation steps of iprodione.

III. To evaluate iprodione and 3,5-DCA removal by inoculation of bacterial inoculum in a grass cover of a biobed.

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# Chapter 2

## Isolation and characterization of bacteria from acidic pristine soil environment able to transform iprodione and 3,5-dichloraniline

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# Isolation and characterization of bacteria from acidic pristine soil environment able to transform iprodione and 3,5-dichloraniline

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## Abstract

Iprodione is a fungicide commonly used in a range of crops resulting in its common detection in natural water resources. Biological degradation constitutes the major dissipation processes controlling its environmental dissipation. We aimed to isolate and characterize soil bacteria able to metabolize iprodione and its key metabolite 3,5-DCA. Initial microcosm laboratory studies in a pristine acidic soil (without previous exposure to the fungicide) showed a slow dissipation of iprodione. Subsequent enrichment cultures from the same soil as well as from a soil with previous exposure to iprodione resulted in the isolation of two bacterial cultures (C2.7 and A1.4) that degraded iprodione and 3,5-DCA respectively, both obtained from the pristine soil. Molecular fingerprinting revealed that C2.7 was composed of two strains, identified via cloning as *Arthrobacter* sp. (strain C1) and *Achromobacter* sp. (strain C2), whereas A1.4 was pure and it was identified as *Pseudomonas* sp. Degradation studies with the purified isolates *Arthrobacter* sp. strain C1, *Achromobacter* sp. strain C2 and their combination in minimal and rich media showed that *Arthrobacter* sp. strain C1 was the key iprodione-degrader, whereas

*Achromobacter* sp. strain C2 was only able to slowly co-metabolize iprodione. Transformation of iprodione by *Arthrobacter* sp. strain C1 resulted in the formation of isopropylamine which could be used by the strain as C and N source. This finding suggests that iprodione degradation by our isolate proceeds via the formation of intermediate metabolites before final hydrolysis to 3,5-DCA. *Arthrobacter* sp. strain C1 showed max iprodione degradation and 3,5-DCA formation at the higher pH-temperature combination (7.5-35°C) and maintained its degradation capacity in the presence of a mixture of pesticides expected to co-occur in on-farm biobed systems. On-going studies will elucidate the metabolic pathway of iprodione by the isolated strains.

**Keywords:** iprodione; 3,5-dichloraniline; biodegradation; *Arthrobacter* sp.; *Achromobacter* sp.; *Pseudomonas* sp.

## 2.1. Introduction

Iprodione [*3-(3,5-dichlorophenyl) N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide*] is a dicarboxamide fungicide commonly used in a wide range of crops to control fungal infestations by *Botrytis cinerea, Alternaria* sp., *Aspergillus* sp., *Penicillium* sp. and *Sclerotinia* sp., (Miñambres *et al*. 2010; Grabke *et al*. 2014). Recently, EU approved iprodione's use extension as a nematicide for the control of *Meloidogyne* sp. in protected crops. In this case iprodione is applied through the irrigation system and precaution should be taken to prevent soil contamination. Iprodione has been classified as probable carcinogen (USEPA, 1998), and it is known to reduce testosterone production, thus delaying male rat pubertal development (Blystone *et al*. 2007). It is not strongly adsorbed onto soil particles with $K_{oc}$ of 400 ml $g^{-1}$ (Stromqvist and Jarvis 2005). As a result, iprodione is relatively mobile in the soil environment and its residues have been detected in surface water (Derbalah *et al*. 2003; Sequinatto *et al*., 2013) and drainage water from golf courses (Ludvigsen *et al*. 2004). Considering all the above, the accumulation of iprodione residues in natural resources could be deleterious for human health and environmental quality and should be mitigated.

Iprodione is moderately persistent in soil with pH being the main factor controlling its dissipation. Walker (1987a) showed a clear trend for increasing rates of dissipation of iprodione in alkaline soils and this was attributed to a combination of abiotic and

microbial hydrolysis by soil bacteria which are favored at alkaline pH. Parallel and follow up studies revealed that repeated applications of iprodione in the same field resulted in a dramatic reduction in its biological efficacy (Entwistle 1986) which was eventually attributed it to the development of enhanced microbial degradation, especially in soils with neutral to alkaline soils (Martin *et al*. 1990; Slade *et al*, 1992; Mitchel and Cain 1996).

Soils exhibiting enhanced biodegradation of iprodione have been used as source for the isolation of iprodione-degrading bacteria. Athiel *et al*. (1995) first reported the isolation of an *Arthrobacter* sp., able to rapidly degrade iprodione. However, little is known regarding the microbial catabolic activities of microorganisms against dicarboxamide fungicides like iprodione in pristine soils, not previously exposed to pesticides. In such soils, the capacity of microorganisms to degrade pesticides depends on the structural similarity of the pesticides to natural compounds encountered by soil microorganisms during their life cycle and used by them as growth substrates (Bollag and Liu 1990). Isolation of pesticide-degrading microbes from such pristine environments has been scarce. However such microbes could act as a valuable genetic pool for the isolation of novel catabolic traits involved in the biodegradation of organic pollutants like iprodione or may carry previously unknown metabolic pathways that are ancestors of currently known pathways found in soil microorganisms isolated from anthropogenically polluted soils.

Soil and *in vitro* studies have shown that 3,5-DCA constitutes the major metabolite of iprodione and of other dicarboxamides (Walker, 1987a; Vanni *et al*., 2000). Indeed previous studies by Mercadier *et al*. (1997) showed that iprodione was microbialy hydrolyzed to 3,5-DCA through the formation of three intermediate metabolites: isopropylamine and 3,5-dichlorophenylcarboxiamide (metabolite I) which are initially produced; the latter is subsequently transformed to 3,5-dichlorophenylurea acetate (metabolite II) which is finally hydrolyzed to 3,5-DCA. This metabolite is rather persistent in soil (PBT Profiler) and has been demonstrated to be a powerful nephrotoxic to rats (Lo *et al*., 1990) and toxic to soil microorganisms (Rochkind *et al*., 1986). Despite that, a number of soil bacteria able to degrade 3,5-DCA have been isolated (Lee *et al*., 2008; Yao *et al*., 2011). Their capacity to transform dichloroanilines produced by the degradation of xenobiotics including dicarboxamides like iprodione is particularly

desirable since their activity would prevent the accumulation of toxic intermediates in soil and minimize the risk for environmental deterioration.

The objective of this study was to isolate and characterize iprodione- and 3,5-DCA degrading bacteria from pristine soils, while previously-exposed soils were also included for comparison purposes. The co-isolation of iprodione and 3,5-DCA degrading bacteria will contribute to the arms-race towards the complete removal of the fungicide and its toxic metabolites in future clean-up strategies. In order to achieve this objectives a series of experiments including initial assessment of the dissipation of iprodione in a pristine soil, isolation of iprodione and 3,5-DCA degrading bacteria via soil enrichment cultures, identification of the isolated bacteria via molecular means and characterization of their metabolic capacities in liquid culture studies.

## 2.2. Material and methods

### 2.2.1. Chemicals

Iprodione, 3,5-DCA, atrazine, chlorpyrifos and isoproturon analytical standards (≥ 97% purity) (Sigma-Aldrich, USA) were used for the preparation of 1000 μg $mL^{-1}$stock solutions in methanol which were utilized only for analytical purposes. Solutions of the above pesticides in DMSO (10000 μg $mL^{-1}$) (Merck, Germany) were prepared, filter sterilized (0.22 μm-pore-size filter; Millipore) and used for the preparation of pesticide-amended growth media. *N*-(1-naphthyl) ethylene diaminedihydrochloride (Sigma-Aldrich, USA) was diluted in deionized water and kept at 4 °C in the dark. Aqueous solutions of piperacillin, vancomycin (10000 μg $mL^{-1}$) and linezolid (500 μg $mL^{-1}$) antibiotics (Sigma-Aldrich, USA) were prepared in sterile deonized water and were used for the preparation of antibiotics-amended growth media.

### 2.2.2. Media

Mineral salts medium (MSM) was prepared as described by Rousseaux *et al*. (2001) and had the following composition (g $L^{-1}$): $K_2HPO_4$ (1.6), $KH_2PO_4$ (0.4). $MgSO_4 \cdot 7H_20$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), salt stock solution (1 mL), vitamin stock solution (1mL), $FeSO_4 \cdot 6H_2O$ stock solution (1 mL, 5.0 g $L^{-1}$). The salt stock solution contained (g $L^{-1}$):

boric acid (2.0), $MnSO_4 \cdot H_20$ (1.8), $ZnSO_4$ (0.2), $CuSO_4$ (0.1), $Na_2MoO_4$ (0.25); the vitamin stock solution was composed of (g $L^{-1}$): thiamin hydrochloride (0.1) and biotin (0.04). In certain cases, MSM was supplemented with 10 ml of a stock solution of sodium citrate (100 g $L^{-1}$) to serve as additional C source (MSM+SC). The stock solutions of vitamins, $FeSO_4 \cdot 6H_2O$ and sodium citrate were filter sterilized (0.22 µm pore size; Millipore) and kept at 4 °C. The pH of MSM was adjusted to 5.0 via addition of HCl prior to autoclaving.Cycloheximide (0.05 g $L^{-1}$) was added to avoid fungal contamination. Luria Bertani (LB) was used for routine cultivation of the isolated bacteria. Solidified MSM and MSM+SC were also prepared by the addition 20 g $L^{-1}$ agar (Difco$^{TM}$) before autoclaving. All media were supplemented with iprodione or 3,5-DCA via addition of appropriate amounts of DMSO stock solutions (10000 µg $mL^{-1}$). In all cases the DMSO percentage in growth media did not exceed 0.2%, which according to preliminary tests did not have any effects on the growth and degrading capacity of the isolated strains.

### 2.2.3. Soils

Two Andisol soils belonging to the Freire series were used in our study: a) a soil without previous exposure to iprodione collected from the rhizosphere of grassland located in the south of Chile, in La Araucanía Region (38°43'60"S and 72°40'00"W) at Maquehue Experimental Station of Universidad de La Frontera and b) a soil with previous exposure to iprodione taken from the rhizosphere of a grassland area in La Araucania region (39°06'00"S and 72°41'00"W) at San Jose orchard near Gorbea (**Figure 2.1**). In order to acquire true rhizosphere soil, ryegrass plants (*Lolium perenne*) growing in the two soils were uprooted and were vigorously shaken, the soil strongly adhered to plant roots (0-0.1 cm) was considered as rhizosphere. Samples were stored in plastic bags at 4 °C and quickly transported to the laboratory for further processing. Soils properties are shown in **Table 2.1**. Upon collection soil samples were partially air-dried, sieved (2 mm) and kept in plastic bags at 4°C until further use. Three subsamples each (10 g) were dried overnight at 110 °C to measure the soil moisture content. At the same time, the water-holding capacity (WHC) was determined by soaking of three soil samples each (30 g) in distillated water for 2 h and then draining overnight according to Priha and Smolander (1999).

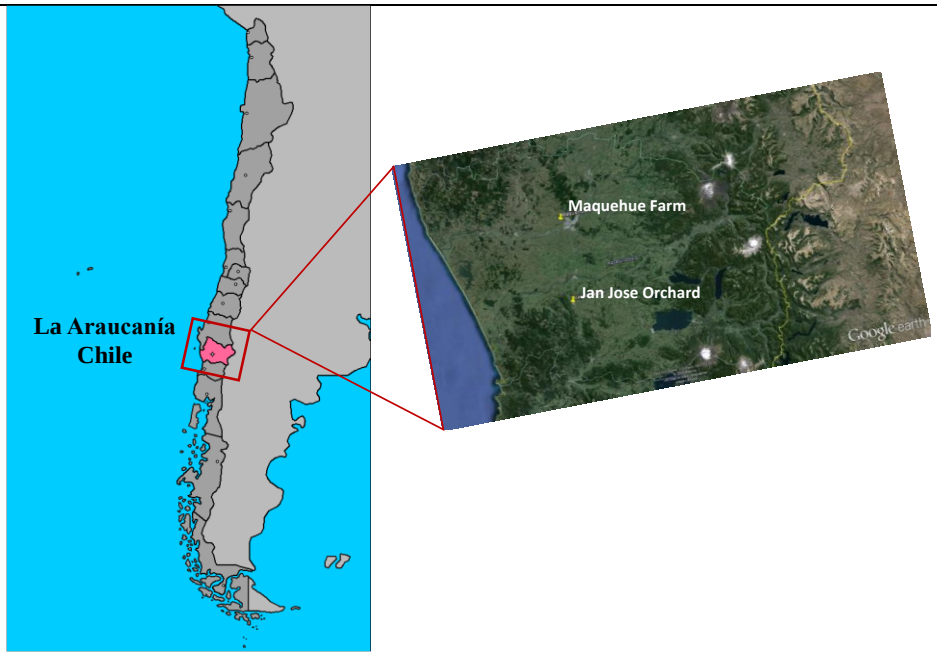


**Figure 2.1.** A schematic map of the location of the two sites from where the soil studied were collected.

**Table 2.1.** Properties of the soils used in the current study

| Soil | Nitrogen[a] (mg $kg^{-1}$ soil dw) | Phosphorus[b] (mg $kg^{-1}$ soil dw) | Potassium[c] (mg $kg^{-1}$ soil dw) | pH[d] | Organic Matter content (%) [e] |
|---|---|---|---|---|---|
| Maquehue | 17 | 12 | 328 | 5.6 | 14 |
| San José | 20 | 11 | 465 | 5.7 | 16 |

[a] Extraction with KCl 2 mol $L^{-1}$ and determined by titration method;
[b] Extraction with sodium bicarbonate solution (0.5 M) at pH 8.5 and determined by blue molybdenum colorimetric method;
[c] Extraction with ammonium citrate (1 M) at pH 7.0 and determined by atomic absorption spectrophotometry;
[d] Determined in soil: water suspensions (1:2.5)
[e] Determined by wet combustion and reduced chromate colorimetric assay

### 2.2.4. Iprodione dissipation in soil

An aliquot of an iprodione solution in methanol (220 µg $mL^{-1}$) was added to triplicate 200 g soil samples from the Maquehue soil, which had not been exposed to iprodione before, to achieve an iprodione concentration in soil of 15.14 mmol $kg^{-1}$. Distilled water was then added to adjust moisture content to 40% of its water holding capacity. The

treated samples were subdivided into 9 samples of 20 g which were placed into aerated plastic bags, incubated in the dark at 20 °C and the moisture content was maintained until the end of assay by regular water additions when needed. Triplicates were removed immediately after application and 3, 7, 14, 21, 42, 56, 70 and 90 d later. Samples were stored at -20°C until extracted and analyzed for iprodione residues and 3,5-DCA formation.

### 2.2.5. Enrichment and isolation of iprodione- and 3,5-DCA-degrading cultures

Iprodione and 3,5-DCA-degrading bacteria were isolated from the two soils mentioned above following enrichment in MSM and MSM+SC media supplemented with iprodione (60 mmol $L^{-1}$). For each soil - media combination, triplicate flasks (250 mL) containing 90 mL of MSM or MSM+SC (+ iprodione or 3,5-DCA) were inoculated with 10 g of each soil and were subsequently incubated on a rotary shaker (150 rpm) for 7 d at 28 °C. A second enrichment cycle was performed by inoculating fresh media with 10 mL from the preceding enrichment cycle. At the end of the second enrichment cycle bacterial colonies were obtained by plating serial dilutions of the final enrichment culture on iprodione- and 3,5-DCA-amended MSM and MSM+SC agar plates. These were incubated at 28° C for 4 days and distinct bacterial colonies were randomly selected according to differences in morphology, pigmentation and growth. All selected colonies were inoculated in fresh LB supplemented with iprodione or 3,5-DCA (30.3 μM) to ensure ample growth and were incubated on a shaking incubator (150 rpm) at 28 °C for 24-48 h. Bacterial biomass was harvested by centrifugation and inoculum levels were adjusted to the spectrophotometrically determined at 600 nm ($OD_{600}$) of 0.05. The generated cell suspension was used for the inoculation of fresh MSM + iprodione or 3,5-DCA (10 mg $L^{-1}$) and was incubated on a shaking platform (150 rpm) at 28 °C for 4d. The degradation of iprodione and the formation/degradation of 3,5-DCA was determined via the colorimetric method of Walker (1987b) with slight modifications. Briefly, 1 mL of each culture suspension was centrifuged (6000 rpm) for 5 min. A 500 μL aliquot of the supernatant was mixed with 2.5 mL of 1M HCl and 250 μL of sodium nitrite solution (20 g $L^{-1}$ in deionized water). The tubes were briefly shaken and allowed to stand for 15 min. 0.25 ml of sulphamic acid solution (100 g $L^{-1}$ in deionized water) were added and the samples were allowed to stand for 10 min with occasional shaking. Finally, 0.5 ml of *N*-(1-naphthyl) ethylene diamine dihydrochloride solution (20 g $L^{-1}$ in

deionized water) were added. The appearance of a magenta color indicated the formation of 3,5-DCA whose concentration was determined spectrophotometrically at 550 nm using an external calibration curve with standard solutions of 3,5-DCA (0.01 to 20 µg $mL^{-1}$). Iprodione-containing cultures showing increasing color intensity compared to the color intensity observed in corresponding non-inoculated controls were considered as positive for the transformation of iprodione. Similarly, 3,5-DCA-amended cultures showing decreasing color intensity compared to the color intensity observed in non-inoculated abiotic controls were considered as positive for the degradation of 3,5-DCA.

### 2.2.6. Degradation of iprodione and 3,5-DCA by selected cultures

The two bacterial cultures (named as C2.7 and A1.4) showing the highest degrading potential were further tested for their degradation kinetics against iprodione and/or 3,5-DCA. Bacteria were initially grown at 28 °C for 48 h in LB broth containing (60 mmol $L^{-1}$) of iprodione or 3,5-DCA to acquire enough biomass for downstream inoculation. Cells were collected by centrifugation (6000 rpm for 10 min), washed and re-suspended in sterile 0.9% NaCl to achieve an $OD_{600}$ of 0.1. Aliquots of those cultures were used to inoculate triplicate iprodione- and 3,5-DCA-amended (60 mmol $L^{-1}$) ) media. For every treatment, non-inoculated abiotic controls were included. All samples were incubated on a shaking platform a 28 °C for 10 days. Chemicals degradation was regularly evaluated by HPLC as described below. Bacterial growth (CFU $mL^{-1}$) was determined on the same days by measuring the $OD_{600}$ and converting it to CFU $mL^{-1}$ using a standard calibration curve approach as previously described by Roehrs *et al*. (2012).

### 2.2.7. Identification of the isolated bacteria

#### 2.2.7.1. DNA extraction and PCR amplification

The two bacterial cultures showing the highest degradation capacity for iprodione (mixed culture C2.7) and 3,5-DCA (pure isolate A1.4, identified as *Pseudomonas* sp. as discussed below) were checked for purity via Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Bacterial DNA was extracted using the PureLink® Genomic DNA kit (Invitrogen, Carlsbad, USA). A nested PCR approach was followed for DGGE analysis. In brief, the almost complete 16S rRNA gene length was amplified using the primers 8f-

1512r as described previously (Felske *et al*., 1997), The PCR product obtained was nested with primers 357f+GC and 534r (Muyzer *et al*., 1993) (194 bp) which was analyzed by DGGE. Amplification was carried out in 25-µl reactions containing 1U of DyNAzyme™ EXT (Finnzymes), 0.2 µM of each primer, 1X buffer (DyNAzyme™ EXT buffer), 1.5 mM of $MgCl_2$, and 200 µM of each dNTPs. The PCR conditions are summarized in **Table 2.2**.

**Table 2.2.** The PCR conditions used for the amplification of the 16S rRNA gene in the different cultures and isolates obtained

| PCR | Primers | Initial denaturation | | Denaturation | | Annealing | | Elongation | | Final Elongation | | Cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | °C | min | °C | Min | °C | min | °C | min | °C | min | N° |
| Initial | 8f - 1512r | 95 | 5 | 95 | 1 | 55 | 1 | 72 | 2 | 72 | 10 | 26 |
| Nested | 357f+GC[a] - 534r | 95 | 5 | 95 | 0.5 | 55 | 0.5 | 72 | 0.33 | 72 | 40 | 26 |
| Colony | 357f+GC[a] - 534r | 95 | 5 | 95 | 0.5 | 55 | 0.5 | 72 | 1 | 72 | 10 | 30 |

[a]A 40-bp GC clamp was added at the 5 end of the forward primers

2.2.7.2. DGGE analysis

DGGE analysis was carried out on an INGENYphorU-2x2system (Ingeny International BV, The Netherlands). Polyacrylamide gels (8%) in 1X TAE buffer were prepared with a denaturating gradient of 45-65% (where 100% denaturant contains 7 M urea and 40% formamide, AppliChem, Germany). Electrophoresis was run for 16 h at 60°C and 75 Volts. Gel was silver stained according to McCaig *et al*., (2001) and the image was captured using a digital camera.

2.2.7.3. Cloning, sequencing and phylogenetic characterization of bacteria

The different members of the bacterial cultures found to degrade iprodione and 3,5-DCA were identified via cloning. PCR products from the initial amplification (almost full length 16S rRNA gene) were purified (Nucleospin II clean-up kit, Macherey-Nagel,

Germany), cloned into the pGEM®-T (Promega, Madison, USA) plasmid vector system and transformed into *Escherichia coli* (DH5a Competent Cells, Invitrogen, USA) following standard procedures (Sambrook *et al*. 1989). Subsequent screening of the clone libraries by PCR-DGGE was carried out as described by Liang *et al*. (2008). White colonies were selected and subjected to colony PCR using primers 341f+GC-534r. Positive clones were screened on a DGGE gel to determine their electrophoretic mobility compared with the band pattern of the starting culture. Representative clones for each band type matching the migration pattern of bands in the starting culture were sequenced. For sequencing, plasmid DNA was extracted and purified from selected colonies using the NucleoSpin Plasmid kit (Macherey-Nagel GmbH, Germany) and sent for sequencing at Cemia S. A., Larissa.

The obtained sequences were compared with the National Centre for Biotechnology Information (NCBI, Maryland, USA) type material nearly full length gene sequences (RefSeq Targeted Loci Project; Bioproject accession number PRJNA33175) with the basic local alignment search tool (BLAST) (Altschul *et al*., 1990) v2.2.30+. Parameters deviating from the default were the e-value (set to a maximum of $10^{-30}$) and the minimum length of subject sequences set to 1200 bp. Sequences were then aligned with Muscle v3.7 (Edgar, 2004) and misaligned parts or non-informative sights were removed using GBlocks v0.9b (Talavera and Castresana, 2007). Finally, maximum likelihood trees were calculated with RAxML v7.4.1 (Stamatakis, 2006) using the general time reversible substitution model for a gamma distribution of site rate heterogeneity and while accounting for invariable sites (GTRGAMMAI). The model was selected according to the JModelTest v3.7 (Darriba *et al*., 2012) Akaike Information Criterion (AIC) scores. 1000 bootstrap replicates were used for calculating the branch support.

### 2.2.8. Purification of iprodione-degrading cultures

In cases where DGGE analysis revealed that the isolated cultures were composed of more than one bacterium, the different bacteria were purified to study their role in the degradation of iprodione. Initial purification was performed by spread plating of the mixed culture on LB agar plates amended with 10 mg $L^{-1}$ iprodione and incubated at 28 °C for 4. Sub-culturing in fresh plates was repeated three more times to achieve purification of the different morphotypes appearing on the plates. Upon sub-culturing

morphologically distinct bacterial colonies were transferred into fresh LB broth + iprodione (10 mg $L^{-1}$) to test their degradation capacity followed by plating on fresh LB + iprodione plates to check purity. To ensure purity, the selected bacteria (isolate C1 identified as *Arthrobacter* sp. and isolate C2 identified as *Achromobacter* sp. as discussed below) were transferred into MSM + iprodione (20 mg $L^{-1}$) which were amended with piperacillin (50 mg $L^{-1}$), a selective antibiotic against gram negative bacteria, or with vancomycin (20 mg $L^{-1}$) and linezolid (10 mg $L^{-1}$) to eliminate gram positive bacteria. For each treatment duplicates were prepared plus abiotic controls. All cultures were incubated on a rotary shaker (150 rpm) at 28 °C for 7 d. The degradation of iprodione in the antibiotic-amended cultures was monitored. Cultivation in antibiotic-amended MSM was repeated 3 more times to ensure complete elimination of the target bacteria. At the end of the fourth cultivation cycle, bacterial biomass was harvested by centrifugation and it was subjected to PCR-DGGE analysis as described above to verify purity.

### 2.2.9. Degradation of iprodione by the isolated bacteria in different media

The degradation capacity of the purified isolates *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2, against iprodione was tested in different growth media aiming to elucidate their role in the degradation of the fungicide and the microbial mechanisms involved (metabolism or co-metabolism). The degradation of iprodione (60 mmol $L^{-1}$) was tested in MSM, MSM+SC and LB where the pesticide constituted the sole C and N source, the sole N source or an extra C and N source. Duplicates per medium where inoculated with the purified isolates *Arthrobacter* sp. strain C1, *Achromobacter* sp. strain C2, their combination (*Arthrobacter* sp. + *Achromobacter* sp.) and the original consortium (C2.7). Duplicate non-inoculated controls were included for all media. The pH of all media was adjusted to 5.0 considering the vulnerability of iprodione to abiotic hydrolysis at pH > 7.0. All cultures were incubated on a shaking platform at 28 °C for 240 h. Degradation of iprodione was measured immediately after inoculation and at 4, 8, 12, 24, 48, 96, 168 and 240 h via HPLC. The strain showing the highest degradation capacity was used for the following assays.

### 2.2.10. Metabolism of iprodione and the formation of isopropylamine by the isolated bacterium

Previous studies by Mercadier *et al* (1997) showed that the first step of the microbial degradation of iprodione results in the formation of 3,5-(dichlorophenyl)-2,4-dioxoimidazolidine and isopropylamine. In the absence of commercial standards for the metabolites reported before, we investigated the formation of isopropylamine during the degradation of iprodione by our isolated bacterium. Thus, triplicate MSM + iprodione (30 mmol $L^{-1}$) cultures were inoculated with *Arthrobacter* sp. strain C1 as described before. Non inoculated controls were also included. The degradation of iprodione and the formation of 3,5-DCA and isopropylamine were determined by HPLC as is described below.

### 2.2.11. Effect of temperature and pH on iprodione degradation by the isolated bacterium

The degrading ability of *Arthrobacter* sp. strain C1 was evaluated in all different combinations of pH (4.5, 6.0 and 7.5) and temperatures (15, 25 and 35 °C). The pH of MSM was adjusted prior to autoclaving by addition of appropriate amounts of NaOH or HCl. For every pH-temperature combination, triplicate inoculated and non-inoculated flasks were prepared. The degradation of iprodione and the formation of 3,5-DCA were measured immediately after inoculation and 24, 48, 96, 168 and 336 h later.

### 2.2.12. The degradation of iprodione and of a mixture of other pesticides by the isolated bacterium

The capacity of *Arthrobacter* sp. strain C1 to degrade iprodione and other pesticides, was evaluated to assess the bioremediation capacity and the spectrum of the degradation capabilities of the isolated strain. The pesticides included in this assay are characterized by different chemical structures compared to iprodione but are used concurrently with iprodione in several crops and consequently are commonly disposed of in on-farm biobed systems. Briefly, triplicate bottles containing MSM supplemented with a mixture of atrazine, chlorpyrifos, isoproturon and iprodione (10 mg $L^{-1}$ each) were inoculated with the iprodione-degrading isolate *Arthrobacter* sp strain C1. Triplicate non inoculated controls were also included to check the abiotic degradation of the pesticides. All samples

were incubated on a rotary shaker (150 rpm) at 28 °C. The degradation of the individual pesticides and cell growth were evaluated regularly by HPLC and curve interpolation, respectively.

### 2.2.13. Pesticide residue analysis

Iprodione and 3,5-DCA were extracted from soil following the method described by Wang *et al*., (2004) with slight modifications. Briefly, 5 g of soil sample were mixed with 10 mL of acetonitrile (ACN) and were shaken (200 rpm) for 1 h at 25 °C. Samples were centrifuged at 10,000 rpm for 10 min, filtered through PTFE membrane (0.22 pore size; Milipore) and directly analyzed by HPLC. The recoveries for iprodione and 3,5-DCA from soil were 94% and 84%, respectively. Pesticides (iprodione, 3,5-DCA, isoproturon, atrazine and chlorpyrifos) were extracted from liquid media by mixing 0.5 ml of the media with 1 ml methanol. The mixture was vortexed and subsequently analyzed by HPLC-UV. The concentrations of isopropylamine in the liquid cultures were determined by its reaction with *p*-nitrophenyl-isothiocyanate to derivate N-isopropyl-(4-nitrophenyl-thiourea) which was then determined by HPLC-UV at 340 nm as described by Mercadier *et al*. (1997). The recoveries for each molecule from media were > 97%.

Pesticide residues were determined in a Marathon III HPLC-UV system equipped with a Grace Smart RP C18 column (150mm x 4.6 mm; Grace, USA). Regarding iprodione and 3,5-DCA degradation assays, those two compounds were eluted using a mobile phase of acetonitrile:water (70:30 v/v) as is described by Carlucci *et al*. (2005). Under these conditions the retention times for iprodione and 3,5-DCA were 4.8 and 3.8 min respectively. Separation of atrazine, isoproturon, chlorpyrifos, iprodione and 3,5-DCA from the mixture of pesticide assay was achieved by a gradient elution program with solvent (A) being acetonitrile and solvent (B) being phosphoric acid 0.1% v/v: 12 min with 50% of A and B, followed by 12 min with 70% of A and 30% of B, finishing with 50% of A and B. In all cases a flow rate of 1 mL $min^{-1}$ was followed and the column temperature was at 30 °C. Under these elution conditions the retention times of iprodione, 3,5-DCA, atrazine, isoproturon and chlorpyrifos were 12.3, 6.6., 5.4, 3.9 and 22.4 min respectively.

### 2.2.14. Data analysis

Data were analyzed by ANOVA parametric test and differences between treatments were detected via the Tukey HSD test at 0.05 level. Statistical analyses were performed with SAS JMP statistical software version 10.0.0.

Four kinetic models were used for fitting the dissipation data of iprodione in soil: the single first order kinetic model and three biphasic models (hockey-stick, first order multi-compartment and double first order in parallel) (FOCUS 2006). The $\chi^2$ test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit. All calculations were made with the statistical package R 3.0.2v.

## 2.3. Results and discussion

### 2.3.1. Iprodione dissipation in soil

The dissipation of iprodione in the not previous exposed Maquehue soil was best described by the single first order kinetics model with the following equation

$$C=C_0e^{-kt}$$

where C= concentration of chemical at time t, $C_0$= concentration of chemical present at time t=0, k = dissipation rate. In general iprodione dissipation relatively slowly (DT50 = 57.4 days, $\chi^2 < 15$, $r^2$ 0.985) and its dissipation was followed by negligible formation of 3,5-DCA (**Figure 2.2**). At a first glance the slow degradation of iprodione observed could be attributed to the lack of previous exposure of the Maquehue soil to the fungicide. However in previous studies in soils with no history of exposure to iprodione DT50s of 23-35 d were observed (Walker *et al*., 1986; Slade *et al*., 1992; Wang *et al*., 2004). The slow dissipation of iprodione in this soil is probably a result of its acidic pH (5.5) compared to all the above studies which were performed in neutral to alkaline soils (pH range of 6.0-8.3). Previous studies have shown that iprodione is prone to hydrolysis at increasing soil pH (Cayley and Hide, 1980), whereas slower dissipation rates have been reported in acidic soils (Walker *et al*., 1986; Walker, 1987a).

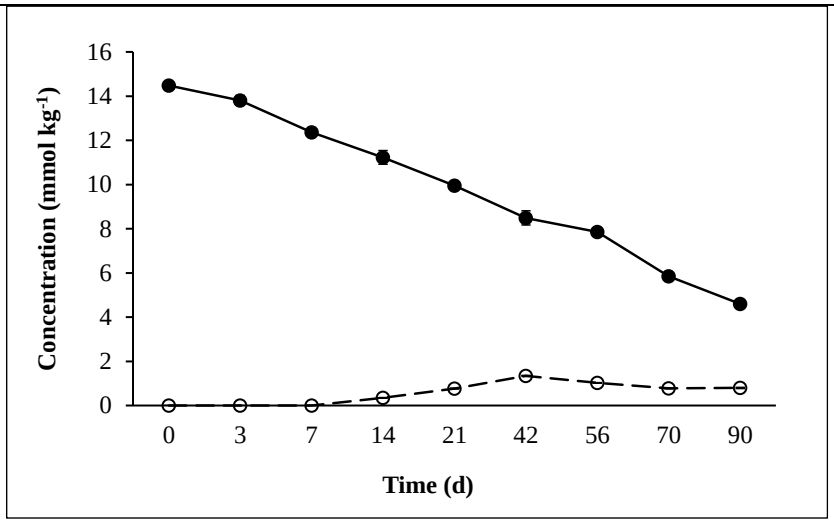


**Figure 2.2**. The dissipation of iprodione (●) and the formation of 3,5-DCA (○, dotted line) in an Andisol soil from Maquehue site without previous exposure to the fungicide. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

### 2.3.2. Enrichment and isolation of iprodione- and 3,5-DCA-degrading bacteria

Based on the slow dissipation of iprodione observed in the Maquehue soil above, and in order to compare and contrast the metabolic capacities of iprodione- and 3,5-DCA-degrading bacteria we decided to use in our enrichments a second soil (San José soil) with known history of exposure to dicarboxamide fungicides including iprodione. From the enrichment cultures, a total of 191 bacteria were selected: 32 from the MSM plus iprodione enrichment (17 from Maquehue and 15 from San José); 25 from MSM plus 3,5-DCA enrichment (15 from Maquehue and 10 from San José); 73 from MSM+SC plus iprodione enrichment (34 from Maquehue and 39 from San José); and 61 from MSM+SC plus 3,5-DCA enrichment (30 from Maquehue and 31 from San José). Out of those isolates only three demonstrated >10% increase in 3,5-DCA formation (C11.6, C2.12 and C2.7), whereas only one, A1.4 showed appreciable decrease in 3,5-DCA concentration (27.8%) (**Table 2.3**). From the above cultures, only the least efficient degrader, C11.6, was obtained from the San José soil which had a history of exposure to iprodione, whereas the most efficient iprodione-degrading cultures C2.7, C2.12 and the most efficient 3,5-DCA-degrading culture A1.4 were all obtained from the not previously exposed Maquehue soil. Despite the low number of pesticide-degrading isolates obtained from this screening, the success ratio obtained from the two soil studied is against the general

belief that soils with extensive pesticides exposure history are more likely to possess a microbial community adapted to the rapid degradation of the given pesticide (Arbeli and Fuentes, 2007). More extensive screening is probably required to verify this further. Previous studies have isolated dicarboxamide-degrading bacteria from previously exposed soils (Athiel *et al*., 1995; Cain and Mitchell, 1996; Mercadier *et al*., 1997). Pesticide-degrading bacteria have been also isolated, but less frequently, by pristine environments with no previous pesticide exposure (Kitagawa *et al*., 2002; Sánchez *et al*., 2004; Nautiyal *et al*., 2008). This has been attributed to the regular exposure of rhizosphere soil microbes to natural aliphatic and aromatic halogenated molecules produced by plants and microorganisms resembling the chemical structure of synthetic pesticides (Gribble, 1992; Copping and Duke, 2007), thus exerting evolutionary pressure for the development of novel xenobiotic catabolic activities by soil microbes (Fetzner, 1998). This is not surprising for iprodione whose imidazolidinedione moiety is common in natural soil substances like allantoin and uric acid. Thus it is expected that amidohydrolases that could cleave this moiety are ubiquitous among soil microorganisms (Burns 1983) and could act as ancestors of novel catabolic pathways.

**Table 2.3.** A list of the initially obtained iprodione- and 3,5-DCA-degrading cultures and their respective degrading performance.

| **Cultures ID** | **Degrading activity[a]** | |
|---|---|---|
| | **Iprodione treatment (% 3,5-DCA increase)** | **3,5-DCA treatment (% 3,5-DCA decrease)** |
| C11.6 | 10.9 ± 0.11 | 0 ± 0.07 |
| A1.4 | 8.5 ± 0.13 | 27.8 ± 0.15 |
| C2.12 | 26.5 ± 0.16 | 0.3 ± 0.09 |
| C2.7 | 54.2 ± 0.36 | 3.8 ± 0.18 |

**[a]** MSM plus iprodione (10 mg $L^{-1}$) cultures showing an increase in the concentration of 3,5-DCA were considered as positive for iprodione degradation, while MSM plus 3,5-DCA (10 mg $L^{-1}$) cultures showing a decrease in the concentration of 3,5-DCA were considered as positive for the dichloraniline degradation. In all cases this initial screening was performed via colorimetric determination of the formation of 3,5-DCA as is described in the materials and methods section.

### 2.3.3. Kinetic degradation of iprodione and 3,5-DCA by selected cultures

Cultures C2.7 and A1.4 showing the highest degradation potential for iprodione and its dichloroaniline derivative respectively were used to determine the degradation kinetics of those compounds. C2.7 completely degraded iprodione in 10 days. Its degradation was followed by the formation of 3,5-DCA which peaked at 6 days post inoculation and slightly decreased thereafter (**Figure 2.3a**). The degradation of iprodione by C2.7 was accompanied by only slight bacterial growth in accordance with findings by Athiel *et al*. (1995) who attributed that to the limited water solubility of this compound. Negligible degradation of iprodione and formation of 3,5-DCA was seen in the A1.4-inoculated and in the non-inoculated cultures (data not shown).

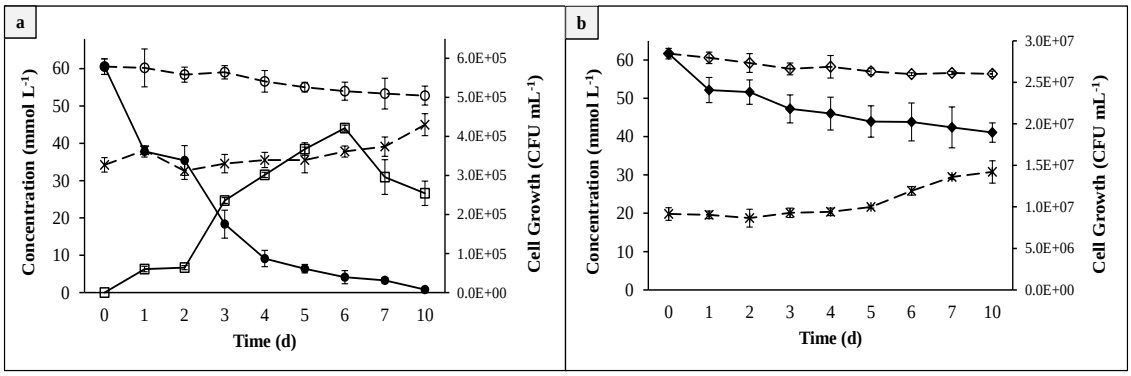


**Figure 2.3.** The degradation of iprodione (a) and 3,5-DCA (b) in MSM by the initially isolated cultures C2.7 (●) and A1.4 (◆). The degradation of the same compounds in the non-inoculated controls is also shown (○,◇, dotted lines). The formation of 3,5-DCA during degradation of iprodione by C2.7 (□) in (a), and bacterial growth of C2.7 and A1.4 is also shown (×,∗ dotted lines). Each value is the mean of three replicates with error bars representing standard deviation of the mean.

Isolate A1.4 (identified as *Pseudomonas* sp. see below) partially degraded 3,5-DCA (*ca*. 34% degradation) after 10 days (Figure 2b) compared to the negligible degradation observed in C2.7-inoculated and in the non-inoculated controls (data not shown). The partial degradation of 3,5-DCA by A1.4 coincided with a gradual bacterial growth. Previous studies have reported the isolation of a range of bacteria identified as *Acinetobacter baylyi* (Hongsawat and Vangnai, 2011), *Bacillus megaterium* (Yao *et al*., 2011) and *Rhodococcus* sp. (Lee *et al*., 2008) which were more efficient degraders of 3,5-

DCA than the *Pseudomonas* sp. strain A1.4. However it should be noted that the degradation by our strain was tested only in MSM, where the chloroaniline constituted the sole C and N source. Previous studies have shown that extra C and/or N sources were necessary to ensure appreciable bacterial growth and achieve effective degradation of 3,5-DCA (Hongsawat and Vangnai, 2011).

### 2.3.4. Bacterial identification and purification of mixed cultures

DGGE analysis of the two isolated cultures C2.7 and A1.4 showed that only the latter was pure, whereas C2.7 comprised of two bands (**Figure 2.4**). Clone libraries, DGGE screening and sequencing of almost the full length of the 16S rRNA gene resulted in the identification of the bacterial members of the isolated cultures. Phylogenetic analysis of the isolate A1.4 showed that it belonged to the genus *Pseudomonas*, lineage and group of *Pseudomonas fluorescens* with the closest relative being a *Pseudomonas baetica* strain (sequence match 99%; **Figure 2.5**). *Pseudomonas* strains are ubiquitous in soil and have been found to degrade several anilines (Konopka *et al*., 1989), chloroanilines (Vangnai and Petchkroh, 2007) and dichloroanilines (Travkin and Golovleva, 2003).

Regarding C2.7, phylogenetic analysis of the amplicon represented by band C1 showed that it belonged to the genus *Arthrobacter* with the closest relative being an *Arthrobacter nicotinovorans* strain (sequence match 99%; **Figure 2.6**). Phylogenetic analysis of the amplicon represented by band C2 showed that it belonged to the genus *Achromobacter* with the closest relative being an *Achromobacter spanius* strain (sequence match 99%; **Figure 2.7**). *Arthrobacter* sp. strains have been previously described as iprodione-degraders (Athiel *et al*., 1995), while bacteria belonging to the genus *Achromobacter* have been reported as degraders of carbofuran (Tomasek and Karns, 1989), methyl parathion (Zhang *et al*., 2005) and endosulfan (Li *et al*., 2009).

Further attempts focused on the purification of the two members of the C2.7 culture and investigation of their role in the degradation of iprodione. Plating and repeated sub-culturing resulted in an initial separation of the two bacterial members. In order to ensure purity of *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2, the two cultures were successively exposed to selective antibiotics. Thus to eliminate *Achromobacter* sp, strain C2, cultures of the *Arthrobacter* sp. strain C1 were amended with the antibiotic piperacillin, acting against Gram-negative bacteria like *Achromobacter* (Almuzara *et al*.,

2010). On other hand, to eliminate *Arthrobacter* sp. strain C1, cultures of the *Achromobacter* sp. strain C2 were amended with vancomycin and linezolid, both active against Gram-positive bacteria (Funke *et al*., 1996; Mages *et al*., 2008). After four subcultures in MSM + antibiotics, DGGE analysis revealed that the two isolates were successfully purified (data not shown).

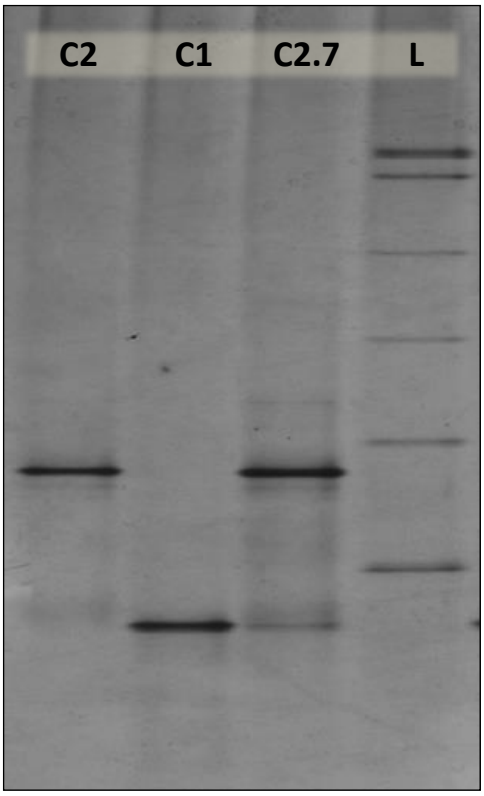


**Figure 2.4.** DGGE analysis of original iprodione-degrading culture C2.7 and of its purified components, *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2. Lane L: Bacterial ladder composed of a mixture of PCR products of the 16S rRNA gene of six bacterial isolates.

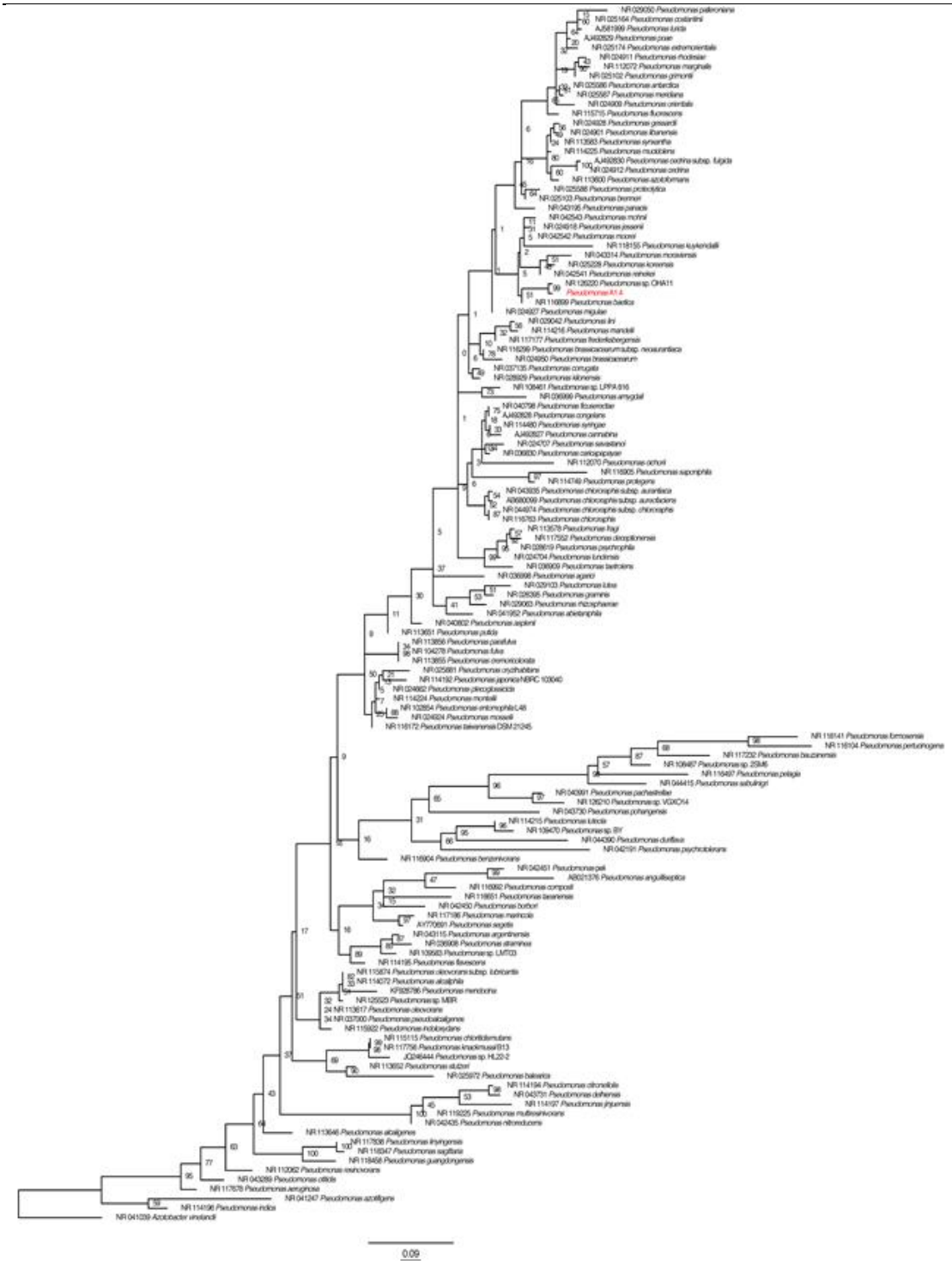


**Figure 2.5.** Phylogenetic analysis of the isolate A1.4, able to partially degrade 3,5-DCA based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with RAxML following the GTRGAMMAI (General Time Reversible with GAMma rate heterogeneity and considering Invariable sites) model. The bootstrap support is expressed in scale from 0 to 100. The NCBI accession numbers of each bacterial type isolate whose sequence was used in the analysis is provided.

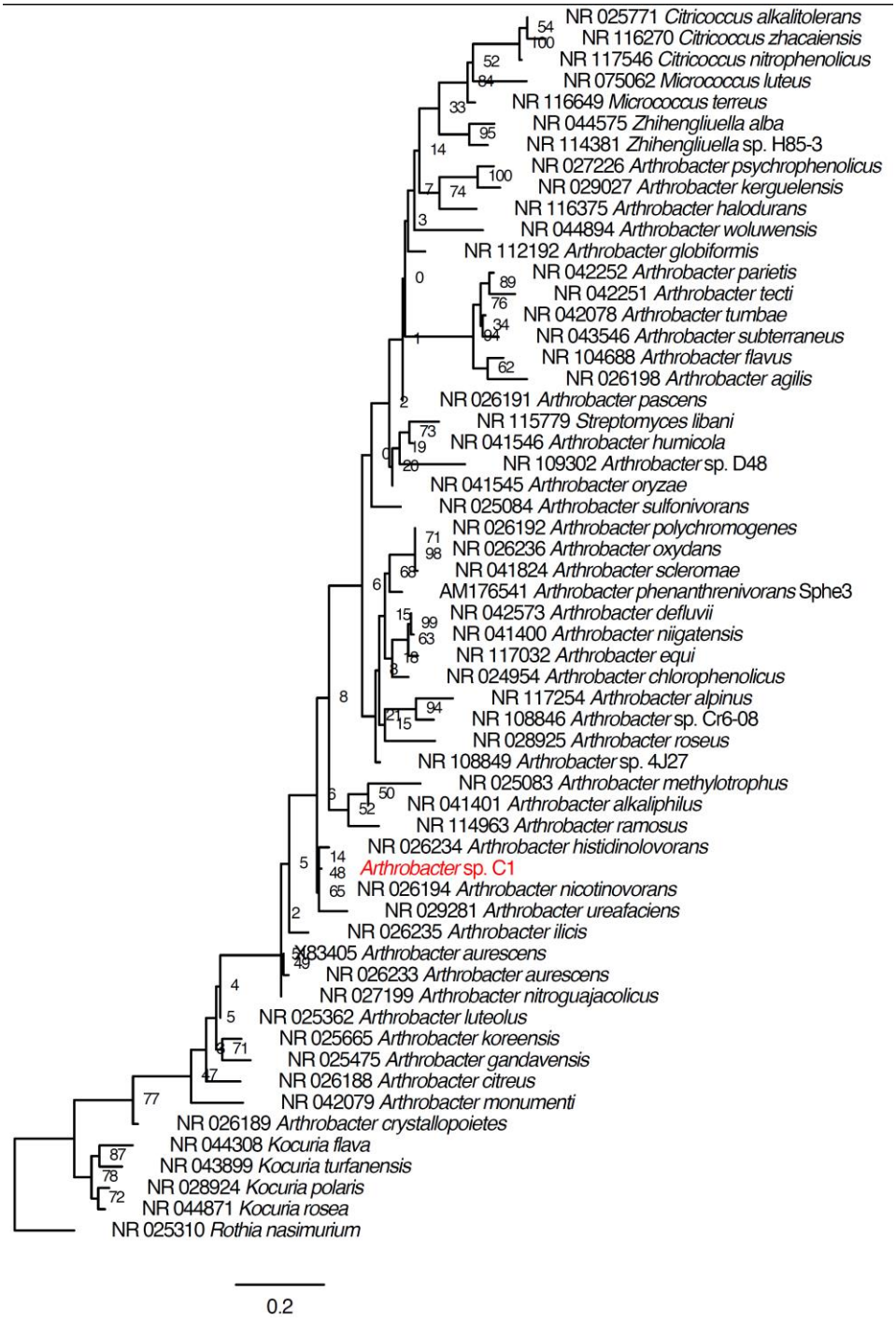


**Figure 2.6.** Phylogenetic analysis of the isolate C1 contained in the culture C2.7 which was able to degrade iprodione based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with RAxML following the GTRGAMMAI (General Time Reversible with GAMma rate heterogeneity and considering Invariable sites) model. The bootstrap branch support is provided in scale from 0 to 100. The NCBI accession numbers of each bacterial type isolate whose sequence was used in the analysis is provided.

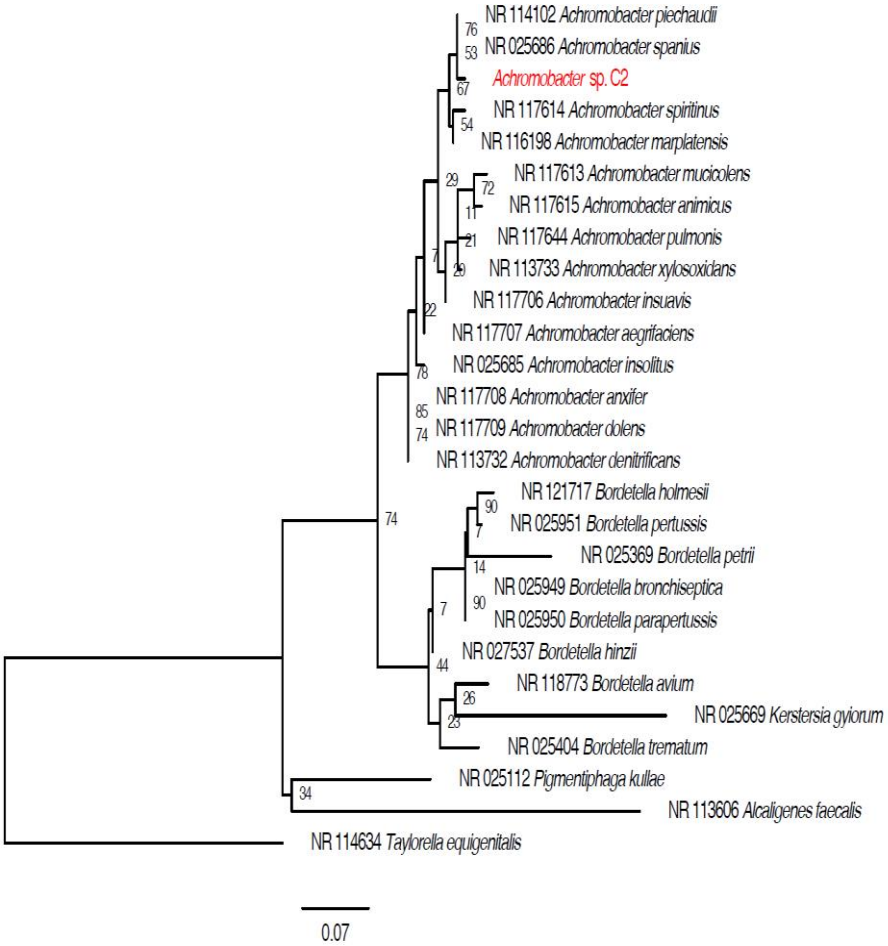


**Figure 2.7.** Phylogenetic analysis of the isolate C2 contained in the culture C2.7 which was able to degrade iprodione based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with RAxML following the GTRGAMMAI (General Time Reversible with GAMma rate heterogeneity and considering Invariable sites) model. The bootstrap branch support is provided in scale from 0 to 100. The NCBI accession numbers of each bacterial type isolate whose sequence was used in the analysis is provided.

### 2.3.5. Degradation of iprodione by the isolated strains in different media

The degradation capacity of the purified isolates *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2 was determined in selective media where iprodione constituted the sole C and N source (MSM), the sole N source (MSM+SC) and in a rich media like LB where iprodione was supplied as an extra C and N source. In a piooneering study Mercadier *et al*. (1997) showed that the degradation of iprodione to 3,5-DCA was only possible with the synergistic action of three *Pseudomonas* isolates: The first two

transformed iprodione to N-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine (metabolite I) and isopropylamine with metabolite I further transformed to 3,5-dichlorophenylurea acetate (metabolite II), while the third isolate was only able to transform metabolites I and II to 3,5-DCA. Based on the above, we hypothesized that the two strains might follow a similar synergistic strategy in the metabolism of iprodione to 3,5-DCA. This hypothesis was tested by comparing the degradation of iprodione and the formation of 3,5-DCA by *Arthrobacter* sp. strain C1, *Achromobacter* sp. strain C2, and their combination (*Arthrobacter* sp. C1 + *Achromobacter* sp. C2). For comparison, the metabolism of iprodione by the original C2.7 was also tested.

The degradation of iprodione by the *Arthrobacter* strain C1 proceeded rapidly in all media with complete degradation observed within 8 and 24 h in the two selective media and LB respectively (**Figure 2.8a**). This rapid degradation was accompanied with 3,5-DCA formation only in LB where the chloroaniline was formed from 48 h onwards when the degradation of iprodione had been completed. On other hand, the *Achromobacter* sp, strain C2 showed no degradation capacity for iprodione in MSM and only limited degradation capacity in MSM+SC, whereas it achieved a complete degradation of iprodione in the LB medium at 240 h (**Figure 2.8b**). These results suggest that the main iprodione-degrading strain in the C2.7 culture was *Arthrobacter* sp. strain C1 which in contrast to the *Arthrobacter* strain reported by Athiel *et al*. (1995) was able to optimally degrade the fungicide at acidic conditions. The late production of 3,5-DCA by *Arthrobacter* sp. strain C1 and only in LB, which favors the growth of this strain, suggests the involvement of a co-metabolic mechanism in the degradation of intermediate metabolites probably produced prior to 3,5-DCA formation (Mercadier *et al*. 1997). On the other hand *Achromobacter* sp. strain C2 is only able to co-metabolize iprodione, so its role in the metabolism of iprodione by C2.7 was not clear.

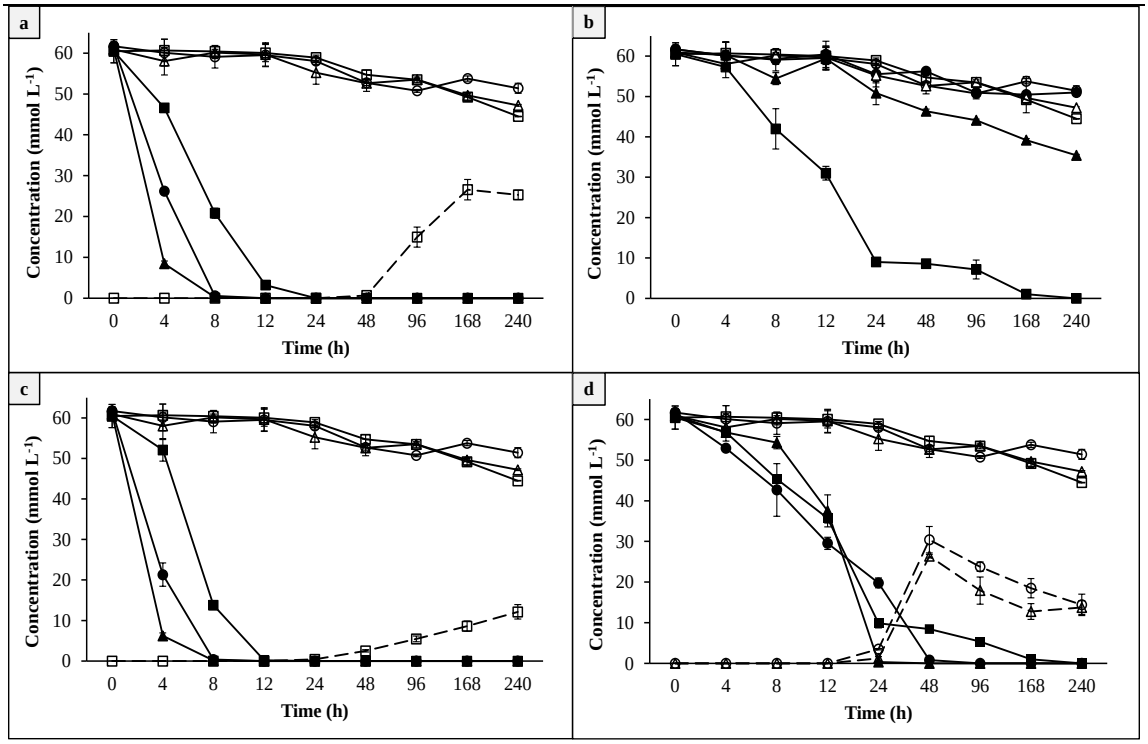


**Figure 2.8.** Degradation of iprodione by *Arthrobacter* sp. strain C1 (a), *Achromobacter* sp. strain C2 (b), *Arthrobacter* sp. strain C1 + *Achromobacter* sp. strain C2 (c) and C2.7 culture (d) in MSM (●,○), MSM+SC (▲, △) and LB (■,□). Inoculated samples (solid lines, closed symbols) and non-inoculated controls (solid lines, open symbols). The formation pattern of 3,5-DCA (dotted lines, open symbols) is also presented. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

The combination of *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2 at the same cell concentration showed degradation patterns similar to *Arthrobacter* sp. strain C1 with rapid degradation of the parent compound accompanied by the late accumulation of 3,5-DCA only in LB (**Figure 2.8c**). Finally, the original C2.7 culture degraded iprodione within 24, 48 and 240 h in MSM, MSM+SC and LB respectively, (**Figure 2.8d**) suggesting a slower degradation compared to *Arthrobacter* sp. strain C1 and *Arthrobacter* sp. strain C1 + *Achromobacter* sp. strain C2. In addition, a different pattern in the formation 3,5-DCA was observed where 3,5-DCA was formed at substantial amounts only in the two selective media with peak concentration observed at 48 h. The discrepancy in the metabolic patterns obtained for *Arthrobacter* sp. strain C1 + *Achromobacter* sp. strain C2 and C2.7, composed by the same two strains, could be attributed to differences in the proportion of the two isolates in the two treatments: strain *Achromobacter* sp. strain

C2 constituted the dominant member of C2.7 as it is demonstrated by the relative intensity of the bands representing those two strains in the DGGE fingerprint of C2.7 (**Figure 2.4**), whereas *Arthrobacter* sp. strain C1 + *Achromobacter* sp. strain C2 was composed of equal proportions of the two strains. This explains the slower degradation of iprodione by C2.7 where *Arthrobacter* sp. strain C1, the key degrader of iprodione, was present at low numbers in the starting inoculum and needed to proliferate in order to degrade iprodione. In the same culture, the earlier formation of 3,5-DCA combined with the initial population dominance of strain *Achromobacter* sp. strain C2 provides indirect evidence for the possible role of this strain in the transformation of intermediate metabolites produced by the degradation of iprodione. However, this remains to be tested. In contrast in *Arthrobacter* sp. strain C1 + *Achromobacter* sp. strain C2 the equal proportion of the two strains in the starting inoculum resulted in the rapid hydrolysis of iprodione by *Arthrobacter* sp. strain C1 and the gradual proliferation of *Achromobacter* sp. strain C2 which reached at population levels able to transform iprodione's intermediate metabolites to 3,5-DCA only in the nutrient rich medium LB. Overall we propose that *Arthrobacter* sp. strain C1 is able to hydrolyze iprodione to intermediate metabolites which are then further metabolized or co-metabolized by strains *Achromobacter* sp. strain C2 and *Arthrobacter* sp. strain C1 respectively to 3,5-DCA.

### 2.3.6. The metabolism of iprodione and the formation of isopropylamine by the isolated bacterium

The rapid degradation of iprodione and the late production of 3,5-DCA by *Arthrobacter* sp. strain C1 suggested the formation of intermediate metabolites before transformation to 3,5-DCA. Considering that our isolate *Arthrobacter* sp. strain was using iprodione as a C and N source, we investigated the formation of isopropylamine, a compound which could be utilized as such nutrient source from our bacterium. Indeed, the rapid transformation of iprodione by *Arthrobacter* sp. strain C1 was accompanied by the concurrent formation of isopropylamine which peaked at 4 h and rapidly degraded to undetectable levels within 10 h (**Figure 2.9**). No 3,5-DCA was formed during the degradation of iprodione, which is in accordance with our findings reported in Figure 4a. In addition, no degradation of iprodione and formation of any of the target metabolites were detected in the non-inoculated control verifying the microbial nature of the transformations observed. These results suggest that the fist metabolic step in the

degradation of iprodione by the *Arthrobacter* sp. strain C1 is in agreement to the pathway proposed by Mercadier *et al*. (1997).

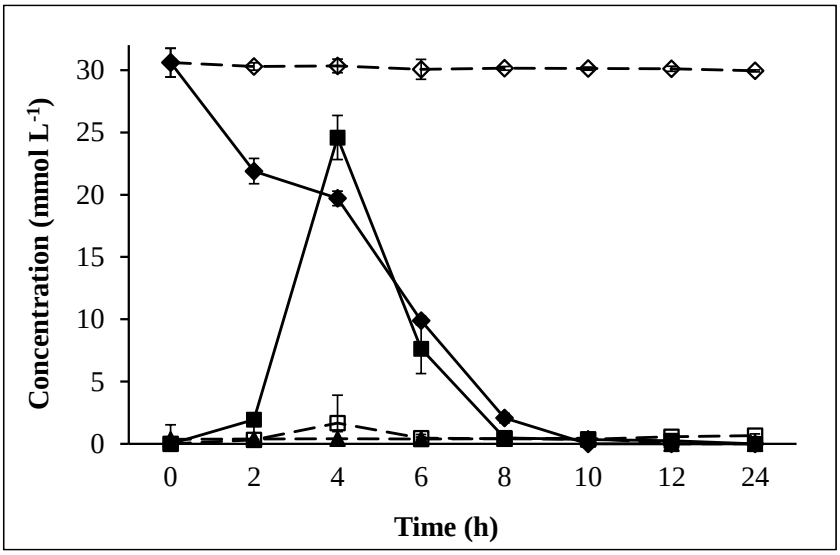


**Figure 2.9.** The transformation of iprodione by *Arthrobacter* sp. strain C1 (◆) in MSM and the formation of isopropylamine (■) and 3,5-DCA (▲). Results in non-inoculated control are also presented (empty captions, dotted lines). Each value is the mean of three replicates with error bars representing standard deviation of the mean.

### 2.3.7. Effect of temperature and pH on iprodione degradation by the isolated bacterium

Considering that the *Arthrobacter* sp. strain C1 was the key iprodione-degrading strain, its capacity to degrade iprodione in a range of pH and temperatures was investigated. In most treatments the degradation of iprodione by the isolated strain was completed in less than 2 days with the exception of the slightly slower degradation observed at the lowest temperature - pH combination (15 °C / pH 4.5) (**Figure 2.10a**). No appreciable degradation of iprodione was evident in the non-inoculated controls at pH 4.5 (**Figures 2.10a, d** and **g**), whereas *ca.* 50 % degradation of iprodione was evident in the non-inoculated samples at pH 6.0 at all temperature levels (**Figures 2.10b, e** and **h**). Iprodione was unstable at pH 7.5 and it was rapidly degraded in the abiotic controls especially at temperatures higher than 25 °C (**Figures 2.10c, f** and **i**). This is in agreement with the well documented vulnerability of all dicarboxamindes to hydrolysis at alkaline pH (Szeto *et al*., 1989). Overall, higher pH and temperatures favored the faster

degradation of iprodione by the *Arthrobacter* sp. strain C1 although at alkaline pH abiotic mechanisms significantly contribute to the degradation of the fungicide.

Regarding the metabolism of iprodione, no 3,5-DCA was formed at pH 4.5 and 6.0 compared to pH 7.5 where the formation of 3,5-DCA in the inoculated cultures increased with increasing temperatures (**Figures 2.10c, f** and **i**). The significant production of 3,5-DCA during degradation of iprodione by *Arthrobacter* sp. strain C1 incubated at the highest temperature - pH combination could be attributed to the more prolific growth of the bacterium under these conditions. This enables the bacterium to rapidly reach at population levels which are needed to co-metabolize the intermediate metabolites of iprodione, as suggested above, and produce 3,5-DCA. In contrast, no 3,5-DCA was detected in the corresponding non-inoculated controls despite the equally rapid degradation of the fungicide. These results suggest the operation of different metabolic pathways of iprodione under biotic and abiotic conditions. This is in agreement with previous studies by Zadra *et al*. (2006) who showed a different metabolic pathway of iprodione in non-inoculated cultures and in cultures inoculated with the fungi *Zygosaccharomyces rouxii*. Our results suggest that pH constitutes a significant factor influencing the production of 3,5-DCA during degradation of iprodione by the *Arthrobacter* sp. strain C1, which was feasible only in neutral to alkaline conditions (pH $\geq$ 6.0) and increased with increasing temperature.

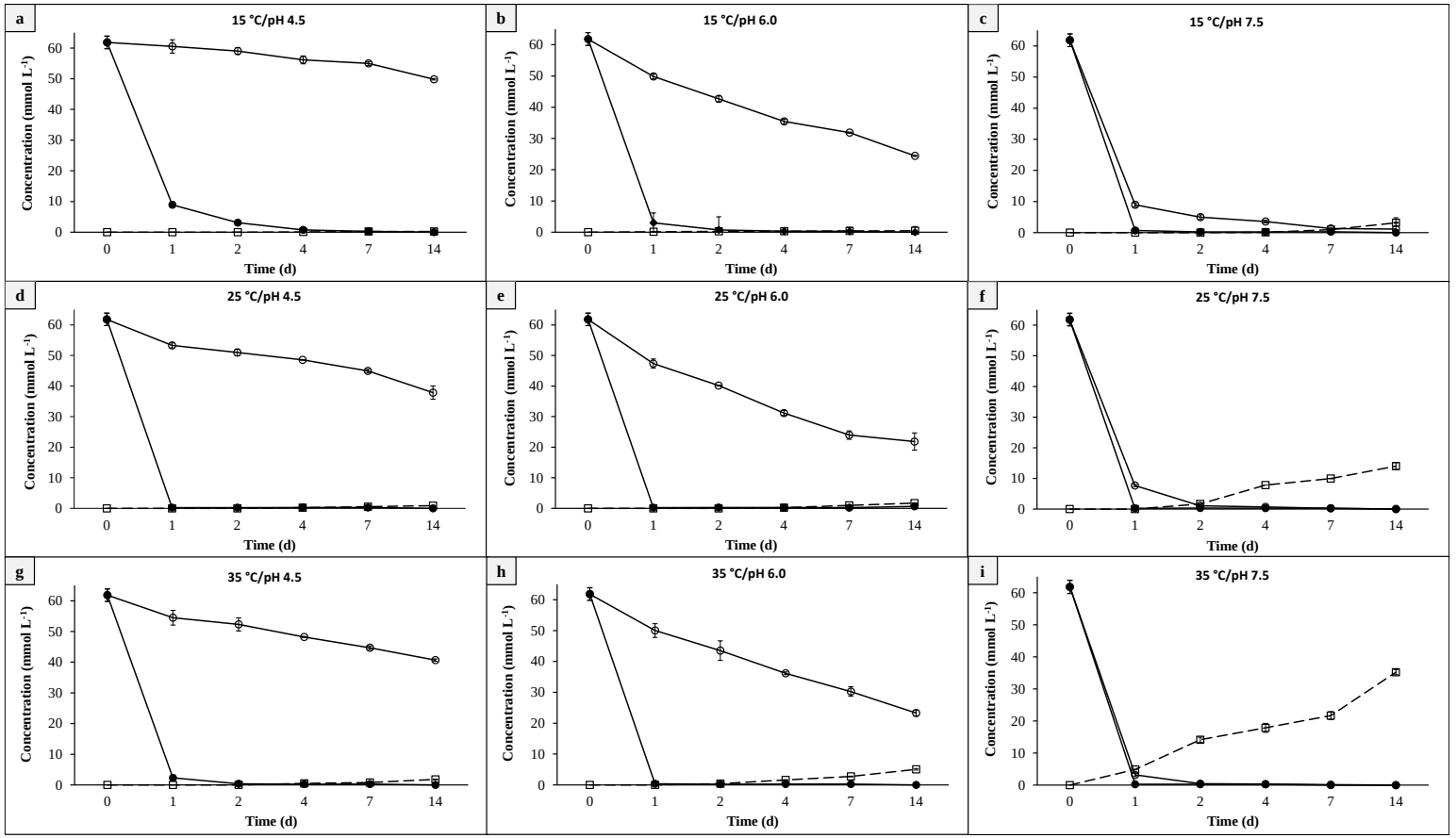


**Figure 2.10.** The degradation of iprodione in MSM inoculated with *Arthrobacter* sp. strain C1 (●) and in non-inoculated controls (○) at different pH and temperatures. The combinations studied were: 15 °C/pH 4.5 (a), 15 °C/pH 6.0 (b), 15 °C/pH 7.5 (c), 25 °C/pH 4.5 (d), 25 °C/pH 6.0 (e), 25 °C/pH 7.5 (f), 35 °C/pH 4.5 (g), 35 °C/pH 6.0 (h) and 35 °C/pH 7.5 (i). The production of 3,5-DCA (dotted line, open square) is also shown. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

### 2.3.8. The degradation of iprodione and of a mixture of other pesticides by strain C1

The degradation of iprodione by *Arthrobacter* sp. strain C1 was investigated in the presence of a mixture of three other pesticides commonly used in combination with iprodione and are expected to be found at high concentrations in on-farm biobed systems. *Arthrobacter* sp. strain C1 was able to rapidly degrade iprodione within 12 h with only slight formation of 3,5-DCA suggesting that the presence of atrazine, chlorpyrifos and isoproturon did not significantly ($p<0.05$) affected its degradation capacity against iprodione (**Figure 2.11**). On the other hand, the strain C1 was not able to transform any of those pesticides. This was not surprising considering the substantially different chemical structure of those compounds compared to iprodione. Several previous studies have documented the high specificity of pesticide-degrading bacteria and their catabolic enzymes (Cullington and Walker 1999; Singh and Walker 2006). Overall, these results suggest that the biodegradation capacity of the *Arthrobacter* sp. strain C1 against iprodione is not expected to be hampered by the co-presence of other pesticides, a characteristic which is considered as a desirable asset for potential bioaugmentation applications.

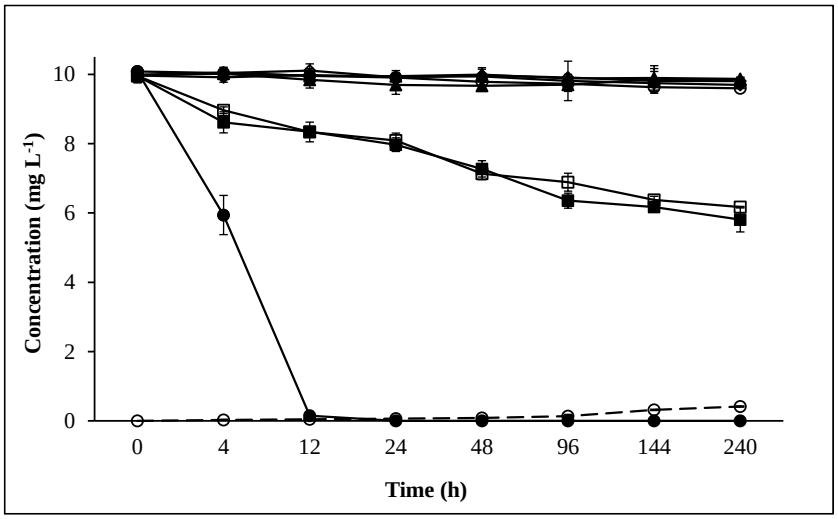


**Figure 2.11**. The degradation of iprodione (●), atrazine (◆), chlorpyrifos (■) and isoproturon (▲) in MSM inoculated by *Arthrobacter* spp. strain C1 and non-inoculated controls (solid lines, open symbols). The formation of 3,5-DCA (dotted line, open circle) by the degradation of iprodione is also shown. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

## 2.4. Conclusions

Iprodione constitutes a very popular fungicide used in a range of crops around the world, with microbial degradation being the key process controlling its environmental dissipation. We report the isolation of an iprodione-degrading culture and of a 3,5-DCA-degrading bacterial strain from an acidic pristine soil with no previous exposure to the fungicide. The latter showed only partial degradation of 3,5-DCA and was identified as *Pseudomonas* sp. The iprodione-degrading culture was composed of two bacterial members identified as *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2 with the former being the key iprodione-degrading member. For the latter, indirect evidence suggest a role in the transformation of intermediate metabolites formed upon the initial hydrolysis of iprodione. The production of intermediate metabolites was further supported by the transient formation of isopropylamine during the degradation of iprodione by the *Arthrobacter* sp. strain C1, with isopropylamine probably used by the bacterium as C and N source. *Arthrobacter* sp. strain C1 maintained its degrading capacity in a wide range of temperature and pH and in the co-presence of a mixture of other pesticides expected to be found along with iprodione in on-farm biobed systems, where effluents by pre- or post-spraying activities are discharged. Further studies will focus on the elucidation of the full metabolic pathway of iprodione by the isolated bacterium and identification of the genetic mechanisms carried by the isolated strain.

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# Chapter 3

## Novel insights into the metabolic pathway of iprodione by soil bacteria

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## Novel insights into the metabolic pathway of iprodione by soil bacteria

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### Abstract

Microbial degradation of iprodione is the key process controlling its soil dissipation. Previously isolated bacteria showed an incomplete capacity to metabolize iprodione to 3,5-dichloraniline (3,5-DCA) which was achieved only by their combination. We recently isolated an iprodione-degrading consortium composed of an *Arthrobacter* sp. strain C1 and an *Achromobacter* sp. strain C2. However the metabolic intermediates and the role of the two strains in the metabolic steps of iprodione were unknown. We examined the degradation and metabolism of iprodione and its possible metabolic intermediates 3,5-dichlorophenyl-carboxamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II) by strains C1, C2, and their combination under selective (MSM) and nutrient rich conditions (LB). In parallel the growth response of those strains during metabolism of the different chemicals was investigated via q-PCR. Furthermore the capacity of strain C1 to transform other pesticides, chemically related to iprodione was investigated. Strain C1 was able to use iprodione and metabolite II as the sole C and N source but only co-metabolized metabolite I. Growth kinetics suggested that the latter was a detoxification step. On other hand, strain C2 was only able to co-metabolize iprodione and its metabolites. Strain C1 degraded

effectively vinclozolin, a structural analogue of iprodione, and partially propanil via production of their respective dichloroanilines. No degradation of procymidone and phenylureas was shown indicating a structure-dependent specificity related to the substituents of the carboxamide moiety. On-going genomic-transcriptomic analysis will identify genes/enzymes involved in the metabolic steps of iprodione.

**Keyword:** Iprodione; microbial metabolism; *Arthrobacter* sp.; *Achromobacter* sp.; vinclozolin

## 3.1. Introduction

Iprodione belongs to the group of dicarboxamide fungicides and it is used for the control of fungal infestations in a range of crops (Miñambres *et al*., 2009; Grabke *et al*., 2014). It is characterized as moderately toxic to small animals (Blystone *et al*., 2007) and probable carcinogenic to humans (USEPA, 1998). Its relatively low $K_{oc}$ of 400 ml $g^{-1}$ denotes a rather weak affinity for soil adsorption (Strömqvist and Jarvis, 2005) suggesting a potential for relatively high mobility in soil. Previous monitoring studies have detected iprodione and its metabolites in drainage water (Ludvigsen *et al*. 2004) and surface water (Sequinatto *et al*., 2013).

Soil pH constitutes the main factor controlling the persistence of iprodione in soil with high degradation rates observed in neutral to alkaline soils (Walker *et al*., 1986). Upon repeated soil applications iprodione is rapidly degraded by adapted soil microorganisms leading to reduced biological efficacy (Martin *et al*. 1990). Studies by Mercadier *et al*., (1996) in adapted and non-adapted soils showed that microbial hydrolysis of iprodione results in the primary formation of 3,5-dichlorophenyl-carboxamide (metabolite I), which is subsequently transformed to3,5-dichlorophenylurea-acetate (metabolite II) and finally to 3,5-dichloroaniline (3,5-DCA). The latter is characterized by relatively high soil persistence (PBT Profiler), high nephrotoxicity to rats (Lo *et al*., 1990) and high toxicity to soil microorganisms (Rochkind *et al*., 1986).

Soils adapted to enhanced biodegradation of iprodione have been used as source for the isolation of iprodione-degrading bacteria. Athiel *et al*., (1995) reported the isolation of an *Arthrobacter*-like strain able to rapidly degrade iprodione to metabolite I with

negligible formation of metabolite II and 3,5-DCA. Follow up studies by Mercadier *et al*., (1997) resulted in the isolation of four *Pseudomonas* strains which were able to metabolize iprodione to 3,5-DCA (through intermediate formation of metabolites I and II) only when combined in a consortium-like process stressing the establishment of synergistic interactions between the *Pseudomonas* isolates to achieve full transformation of iprodione to 3,5-DCA. Recent studies by Campos *et al*., (2015) reported the isolation of an iprodione-degrading consortium composed, as depicted by molecular fingerprinting analysis, by an *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2 which were subsequently purified. The former strain was identified as the key-degrader of iprodione and it was able to transform iprodione through transient formation of isopropylamine which was utilized by the bacterium as C and N source, while the latter was able to transform iprodione only in the presence of alternative C and N sources with no formation of isopropylamine. The production of isopropylamine by strain C1 provided evidence for the operation of a metabolic pathway similar to the one reported by Athiel *et al*. (1995). However, the formation of the intermediate metabolites was not analytically detected and the contribution of the two bacteria in the different steps of iprodione metabolic pathway was not elucidated.

In the present study, we aimed to fully elucidate the metabolic pathway of iprodione by the two purified strains C1 and C2 and identify their role in the transformation steps of iprodione. To achieve this, the metabolism of iprodione, metabolite I and metabolite II by the two individual bacteria and their combination was explored under selective and nutrient rich growth conditions. In parallel measurement of the growth of the two bacteria during the degradation of the studied chemicals by strain-specific q-PCR analysis provided information on the nature (co-metabolism vs catabolism) of the different metabolic steps. Further evidence for the metabolic capabilities of the isolated strains were obtained by exploring their ability to degrade pesticides sharing common chemical structures with iprodione like carboxamide bonding.

## 3.2. Materials and methods

### 3.2.1. Chemicals

Diuron, iprodione, isoproturon, linuron, procymidone, propanil, vinclozolin, 3,4-DCA, 3,5-DCA, (≥ 97% purity), *para*-nitrophenyl-isothiocyanate and 3,5-

dichlorophenyl isocyanate (96% purity) were purchased from Sigma-Aldrich, USA. Stock solutions of pesticides and their metabolites in methanol (1000 µg $mL^{-1}$) were prepared and used for analytical purposes. Solutions of the above chemicals in DMSO (10,000 µg $mL^{-1}$) (Merck, Germany) were prepared, filter sterilized (0.22 µm-pore-size filter; Millipore) and used for the preparation of growth media amended with these chemicals.

### 3.2.2. Growth media

The nutrient-rich medium Luria Bertani (LB) and a mineral salt medium (MSM) were used in the current study. The latter was prepared as described by Rousseaux *et al*., (2001) but without the addition of sodium citrate as C and N source. The pH of both media was adjusted to 5.0 by addition of HCl to minimize alkaline hydrolysis of iprodione. Media were supplemented with the corresponding pesticide or metabolite by addition of appropriate amounts of sterile DMSO stock solutions (10,000 µg $mL^{-1}$).

### 3.2.3. Bacterial strains

Two bacterial strains *Arthrobacter* sp. C1 and *Achromobacter* sp. C2 purified from an iprodione-degrading consortium isolated from a pristine soil were utilized in the current study (Campos *et al*. 2015). Bacteria were initially grown in LB amended with iprodione (60 mmol $L^{-1}$) at 28 °C for 48 h to obtain enough bacterial biomass for their subsequent use as inoculum. Bacterial pellet was collected by centrifugation at 6000 rpm for 10 min, washed 3 times with sterile dd$H_2O$ and re-suspended in a sterile 0.9% NaCl solution. Aliquots from the cell suspension were used to inoculate triplicate flasks of MSM or LB amended with the studied chemical (60 mmol $L^{-1}$) to a spectrophotometry amount at 600 nm of 0.2.

### 3.2.4. Synthesis of iprodione metabolites

Synthesis of metabolites I and II was performed as described by Athiel *et al*., (1995) with modifications, in the preparation method of metabolite II. Specifically, metabolite II was synthesized as follows: 3,5-dichlorophenylisocyanate (1,880 mg) was added dropwise, under stirring to a solution of 750 mg of glycine (0.01 M) in 7 mL of NaOH (0.75 M). The mixture was heated to 30 °C with stirring for 4 h, until the complete

formation of a white precipitate, left at 4ºC overnight, the precipitate was filtered through a Gooch funnel and washed with $H_2O$ (3 ml). The solution was acidified (5ml HCl, 1N) at 0 °C. Finally, the solid precipitate was collected by filtration and dried under vacuum overnight to obtain 865 mg of metabolite II (synthesis yield 36%), and the melting point was 199-201 ºC. The NMR profile $^1$HNMR ($CDCL_3$, DMSO d6): δ 3.92 (2H, d, $CH_2$); 5.41 (2H, s, NH); 6.88 (1H, s, arom); 7.41 (2H, s, arom); and 8.89 (1H, s, COOH) ppm verified the purity of the molecule synthesized. A proportion of metabolite II (350 mg) was dissolved in 10 ml HCL (4 N) and refluxed with stirring for 2 h to synthesize metabolite I. After cooling the solution was filtered and the solid precipitate (260 mg), was metabolite I (80% synthesis yield), melting point 197-199 ºC. NMR analysis verified the purity of the synthesized molecule; $^1$HNMR ($CDCL_3$, DMSO d6); δ 4.16 (2H, s, $CH_2$); 7.38 (2H, s, arom); and 7.43 (1H, s, arom) ppm.

### 3.2.5. Metabolism of iprodione and its metabolites by bacterial isolates

The capacity of strains C1 and C2, and of their combination (inocula ratio 1:1) to metabolize iprodione and its metabolites I and II was investigated in MSM and LB. Triplicate flasks of LB and MSM amended with iprodione (60 mmol $L^{-1}$) were inoculated with strains C1, C2, and their combination (C1 + C2) as described above. Triplicate non inoculated flasks for each medium were also prepared. All samples were incubated on a shaking platform at 28 °C for 240 h. Degradation of iprodione and formation of isopropylamine, metabolite I, metabolite II and 3,5-DCA were measured immediately after inoculation and at 4, 8, 12, 24, 48, 168 and 240 h by HPLC-DAD as described below. In parallel aliquots from the bacterial cultures co-inoculated with strains C1 and C2 were removed at 0, 8, 24, 48 and 240 h and the abundance of the two bacterial strains was determined via q-PCR analysis as described below.

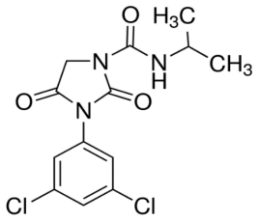
The exact same experimental procedure was repeated for metabolite I (100 mmol $L^{-1}$) and metabolite II (110 mmol $L^{-1}$) to verify the capacity of the same bacterial inocula to metabolize putative intermediate metabolites of iprodione and identify the nature of the transformation process (catabolic vs co-metabolic). Thus, degradation of metabolite I and its transformation to metabolite II and 3,5-DCA (metabolite I experiment), and degradation of metabolite II and formation of 3,5-DCA (metabolite II experiment) were measured at 0, 4, 8, 12, 24, 48, 120, 192 and 288 h. The abundance of bacterial strains

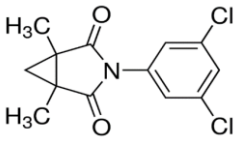
C1 and C2 was determined in the combined inoculation treatment via q-PCR at 0, 12, 48 and 192 h.

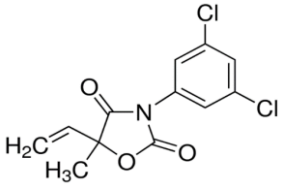
### 3.2.6. Degradation of pesticides with similar chemical structure to iprodione

The ability of *Arthrobacter* sp. strain C1 to degrade other pesticides with similar chemical structure to iprodione was also investigated. The chemical structures of the pesticides tested are shown in **Figure 3.1.**

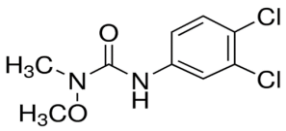
**Dicarboxiamide fungicides**

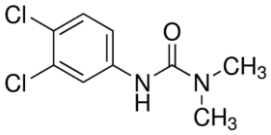

Iprodione

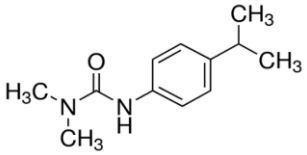

Procymidone


Vinclozolin

**Phenylurea herbicides**


Linuron


Diuron


Isoproturon

**Acetanilide herbicide**

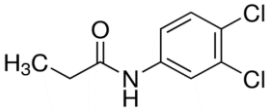

Propanil

**Figure 3.1.** The chemical structures of the pesticides tested in the current study for the capacity of iprodione-degrading strain *Arthrobacter* C1 to metabolize them.

Triplicate flasks containing MSM amended with vinclozolin (35 mmol $L^{-1}$), procymidone (35 mmol $L^{-1}$), isoproturon (48.5 mmol $L^{-1}$), diuron (43 mmol $L^{-1}$), linuron (40 mmol $L^{-1}$) and propanil (46 mmol $L^{-1}$) were inoculated with strain C1 as described before. Triplicate non inoculated flasks for each pesticide studied were also prepared. All cultures were incubated in a shaking incubator at 180 rpm and 28 °C for 480 h. Degradation of pesticide and formation of dichloroaniline metabolites 3,5-DCA and

3,4-DCA were measured immediately after inoculation and at 48, 96, 168, 240, 360 and 480 h by HPLC-DAD. Bacterial growth (CFU $mL^{-1}$) was determined for the same measured points by spectrophotometric determination and interpolation to CFU $mL^{-1}$ using a standard calibration curve as described by Roehrs *et al*., (2012).

### 3.2.7. Determination of bacterial abundance via q-PCR

Bacterial biomass from aliquots (1.5 ml) of the liquid cultures co-inoculated with strains C1 and C2 was harvested by centrifugation at max speed for 2 min. The pellet was processed for DNA extraction using the PureLink® Genomic DNA kit (Invitrogen, Carlsbad, USA) according to the manufacturers' instruction. The concentration and purity of the DNA was measured by the Qubit dsDNA BR assay kit using a Qubit® 2.0 Fluorometer (life technologies).

Specific primers sets for the two isolates studied were designed to follow their abundance during degradation of iprodione and/or their metabolites. Primers were analyzed with the PrimerSelect™ software (Lasergene®, DNASTAR) for undesired secondary structures formation and their specificity was first checked *in silico* with the online tool Primer-BLAST (http://ncbi.nlm.nih.gov/tools/primer-blast/). The sequences and characteristics of the primers used are shown in **Table 3.1**. The specificity of the two primer sets was further verified *in vitro* as follows: DNA obtained from a mixed culture of strains C1 and C2 grown in LB was subjected to PCR separately with the two sets of primers. Amplification was carried out in 25-µl reactions containing 1U of DyNAzyme™ EXT (Finnzymes), 0.2 µM of each primer, 1X buffer (DyNAzyme™ EXT buffer), 1.5 mM of $MgCl_2$, and 200 µM of each dNTPs. The thermocycling conditions were as follow: 5 min at 95 °C of initial denaturation, 30 cycles of 30 sec at 95 °C, 30 sec at 56 °C, 2 min at 72 °C, followed by final elongation step of 10 min at 72 °C. The PCR products amplified were run on an 1% (w/v) agarose gel to confirm their size and purity and further purified (Nucleospin II clean-up kit, Macherey-Nagel, Germany), cloned to the pGEM®-T(Promega, Madison, USA) plasmid vector system, and transformed into *Escherichia coli* (DH5a Competent Cells, Invitrogen, USA) according to standard procedures (Sambrook *et al*., 1989). White colonies containing the right insert were selected for plasmid extraction using the NucleoSpin Plasmid kit (Macherey-Nagel GmbH, Germany). Plasmid DNA was quantified in Qubit® 2.0 Fluorometer and sent for sequencing (Cemia S. A., Larissa, Greece). The sequences

obtained were aligned with the original 16s rDNA sequence of the two strains which were used for primers design and showed 100% match.

**Table 3.1.** Properties of the primer sets designed to follow via q-PCR the growth of two bacterial strains C1 and C2 during degradation of iprodione and its derivatives

| Target strain | Primers | Sequence (5' – 3') | Sequence Position | Amplicon Size (bp) | Annealing Temperature (°C) |
|---|---|---|---|---|---|
| *Arthrobacter* sp. C1 | Arth_f | CAGCACGGACTTCGGTCTGGTG | 44 - 65 | 158 | 58 |
| | Arth_r | AAGACCTTGCACTATTAGAGCGGC | 178-201 | | |
| *Achromobacter* sp. C2 | Achr_f | GAACCGGAAAGACCTGGAAACAG | 948-970 | 237 | 58 |
| | Achr_r | TCTTGGGCTTCACGCATGCT | 1165-1184 | | |

q-PCR was performed in 15 µL reaction volumes containing 1X KAPA SYBR® FAST qPCR Master Mix (2X) Universal, 1 µM of each primer, and *ca*. 0.2 ng DNA. The thermocycling program used was as follows: 3 min at 95 ºC; 40 cycles of 15 sec at 95 ºC, 20 sec at 58 ºC, 11 sec at 72 ºC; followed by a melting curve to check the specificity of the products. All q-PCR were performed on a Mx3000Psystem (Stratagen, UK). The copy numbers of the target genes were determined by external calibration curve (Rousidou *et al*. 2013) using the recombinant plasmids described above. q-PCR efficiencies for the different assays ranged from 85.5 to 99% with $r^2$values from 0.994 to 0.997.

### 3.2.8. Pesticides analysis

Pesticides and metabolites were extracted from liquid cultures by mixing 0.5 mL of culture with 1 mL of acetonitrile. The mixture was vortexed, centrifuged and injected into a Shimadzu HPLC-PDA system equipped with a Shimadzu, VP-ODS, C18 column (4.6 mm x 150mm). The formation of isopropylamine during degradation of iprodione was determined by derivatization according to Mercadier *et al*., (1997). Briefly isopropylamine reacts with *para*-nitrophenyl-isothiocyanate leading to the formation of *N*-isopropyl-(4-nitrophenyl-thiourea) which could be measured by HPLC. Iprodione, derivatized isopropylamine, vinclozolin, procymidone and 3,5-DCA were eluted using a

mobile phase of acetonitrile:water (70:30 v/v) as described by Carlucci *et al*., (2005), with retention times of 4.8, 2.8, 5.3, 4.2 and 3.8 min, respectively. For metabolites I and II, a mobile phase of acetonitrile:water acidified to pH 2.6 with acetic acid (32:68 v/v) was necessary to achieve satisfactory separation with retention times of 11.1 and 12 min, respectively. For separation of isoproturon, diuron and linuron, a mobile phase of acetonitrile:water:phosphoric acid (75:25:0.25 v/v) was used with retention times of 2.5, 3.2 and 3.4 min, respectively (Turnbull *et al*., 2001). Finally, for the analysis of propanil and 3,4-DCA, a methanol:water (80:20 v/v) mobile phase was utilized and the two chemicals eluted with retention times of 6.1 and 5.3 min respectively (Zhang *et al*., 2011). All analyses were performed at a flow rate of 1 mL $min^{-1}$ with a column temperature of 30 °C. The recoveries for each molecule ranged between 98.5% and 102.7%.

### 3.2.9. Data analysis

Data were analyzed by ANOVA parametric test using analysis of means via Tukey HSD test at 0.05 level. Statistical analyses were performed with SAS JMP statistical software version 10.0.0.

$DT_{50}$ values of pesticides and iprodione metabolites were calculated using the single first order (SFO) kinetic model. In cases where the SFO did not provide an acceptable fit to the measured data the biphasic hockey-stick (HS) model was used (FOCUS, 2006). The $\chi^2$ test as well as visual inspection and the distribution of the residuals were used as criteria to assess the model providing the best fit to the measured data. All calculations were made with the statistical package R 3.0.2v.

## 3.3. Results

### 3.3.1. The degradation and metabolism of iprodione

The degradation of iprodione in both media was best described by the SFO kinetic model, except for strain C2 grown in LB where the biphasic HS model provided the best fit to the measured data. In general, variations in the degradation of iprodione by the two bacteria in the different media were observed (**Figure 3.2**). A significantly faster degradation of iprodione by strain C1 was evident. Thus the lowest $DT_{50}$ values

(2.3 and 3.8 h in MSM and LB, respectively) were observed in cultures inoculated with strain C1 followed by cultures co-inoculated with strains C1 and C2 (4.1 and 4.1 h, respectively), while the slowest degradation was observed by strain C2 which degraded iprodione only in LB ($DT_{50}$=19.5 h).

We further investigated the metabolic pathway of iprodione by the two isolates under different nutritional regimes. Thus in MSM inoculated with strain C1, either alone or in combination with strain C2, iprodione metabolism proceeded via transient formation of isopropylamine and production of metabolite I which accumulated in the medium (**Figures 3.2a** and **3.2c**). In contrast in LB strain C1, either alone or in co-culture with strain C2, hydrolyzed iprodione to metabolite I (with no parallel formation of isopropylamine) which was this time further transformed to metabolite II and finally to 3,5-DCA (**Figures 3.2d** and **3.2f**). On the other hand, strain C2 metabolized iprodione to 3,5-DCA in LB (no metabolism in MSM, **Figure 3.2b**) with the intermediate formation of metabolites I and II (**Figure 3.2e**).

Degradation of iprodione in MSM did not appear to stimulate the growth of the two strains which showed a significant decrease ($p<0.05$) in their abundance from 8 h onwards (**Figure 3.2g**). A different growth response by the two strains was observed in LB with strain C2 showing a relatively stable population throughout the incubation compared to strain C1 whose population significantly declined ($p<0.05$) at 24 h followed by a gradual increase from then onwards, although its abundance did not reach to the levels observed at 8 h (**Figure 3.2h**).

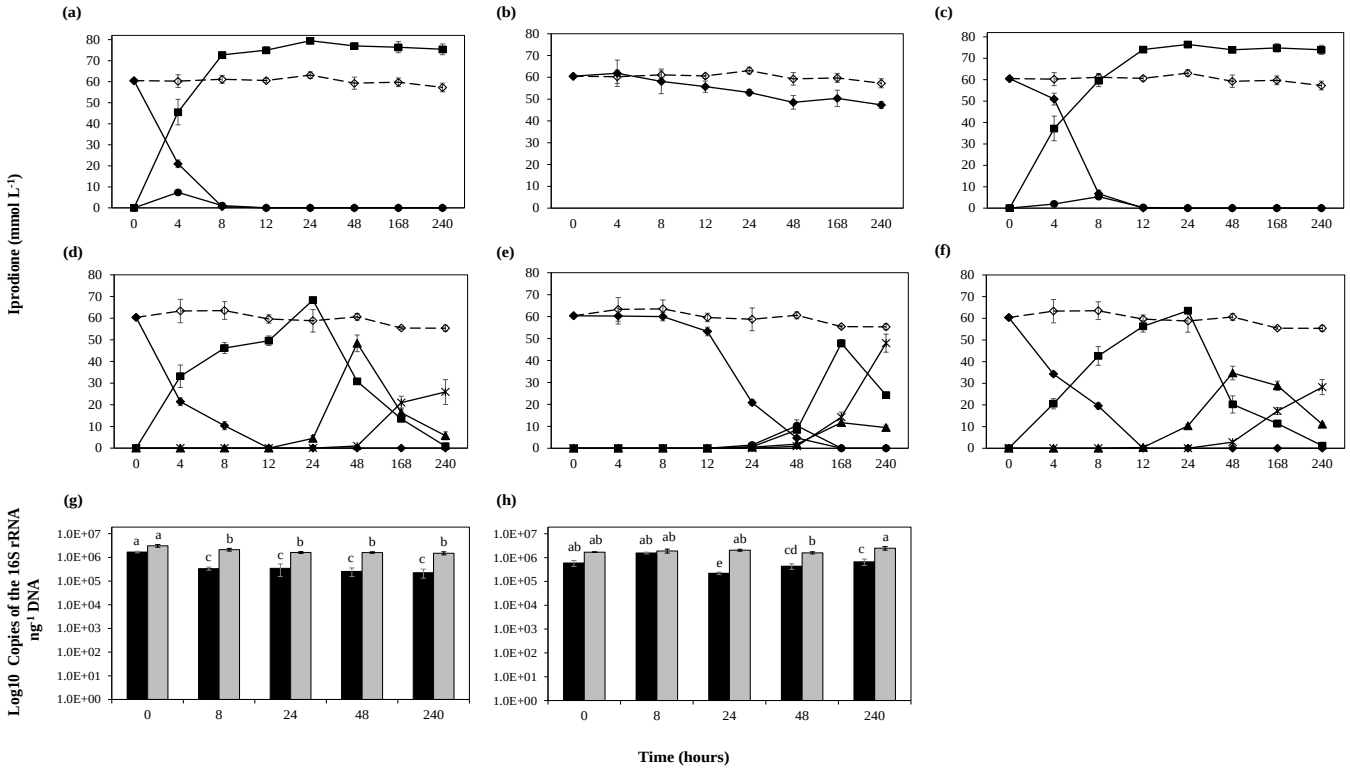



**Figure 3.2.** Degradation of iprodione (◆) and formation of isopropylamine (●), metabolite I (■), metabolite II (▲) and 3,5-DCA (∗) in MSM (a, b, c) and LB (d, e, f) inoculated with strains *Arthrobacter* sp. C1 (a, d), *Achromobacter* sp. C2 (b, e) and their combination (c, f). The degradation of iprodione in non-inoculated cultures is also presented (◇, dotted lines). The growth of strains *Arthrobacter* sp. C1 (black bar) and *Achromobacter* sp. C2 (grey bar) co-inoculated in MSM (g) and LB (h) were determined by q-PCR quantification of their 16S rRNA genes. Bars followed by the same letter are not statistical different at the 5% level. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

### 3.3.2. The degradation and metabolism of metabolite I

In line with the metabolic patterns obtained with iprodione, the two strains failed to degrade metabolite I in MSM (**Figures 3.3a, b** and **c**). On the other hand, both bacterial strains and their combination were able to degrade metabolite I in LB and the degradation patterns observed followed first order kinetics. Strain C2 and its combination with strain C1 showed the more rapid degradation of metabolite I ($DT_{50s}$ of 39.7 and 43.1 h respectively), compared to strain C1 which degraded metabolite I at a slower rate ($DT_{50}$= 71 h). No appreciable degradation of metabolite I was observed in the abiotic control in both media.

The transformation of metabolite I proceeded in a similar way in all treatments via formation of metabolite II which was further hydrolyzed to 3,5-DCA. The latter accumulated in the growth media (**Figures 3.3d, e** and **f**). During degradation of metabolite I the two strains C1 and C2 showed variable growth patterns when co-inoculated in LB. Thus strain C1 showed a drastic reduction ($p<0.05$) in its abundance up to 48 h post inoculation followed by a recovery by 192 h (**Figure 3.3g**). In contrast strain C2 maintained a stable population during the first 48 h followed by a significant increase ($p<0.05$) at 192 h.

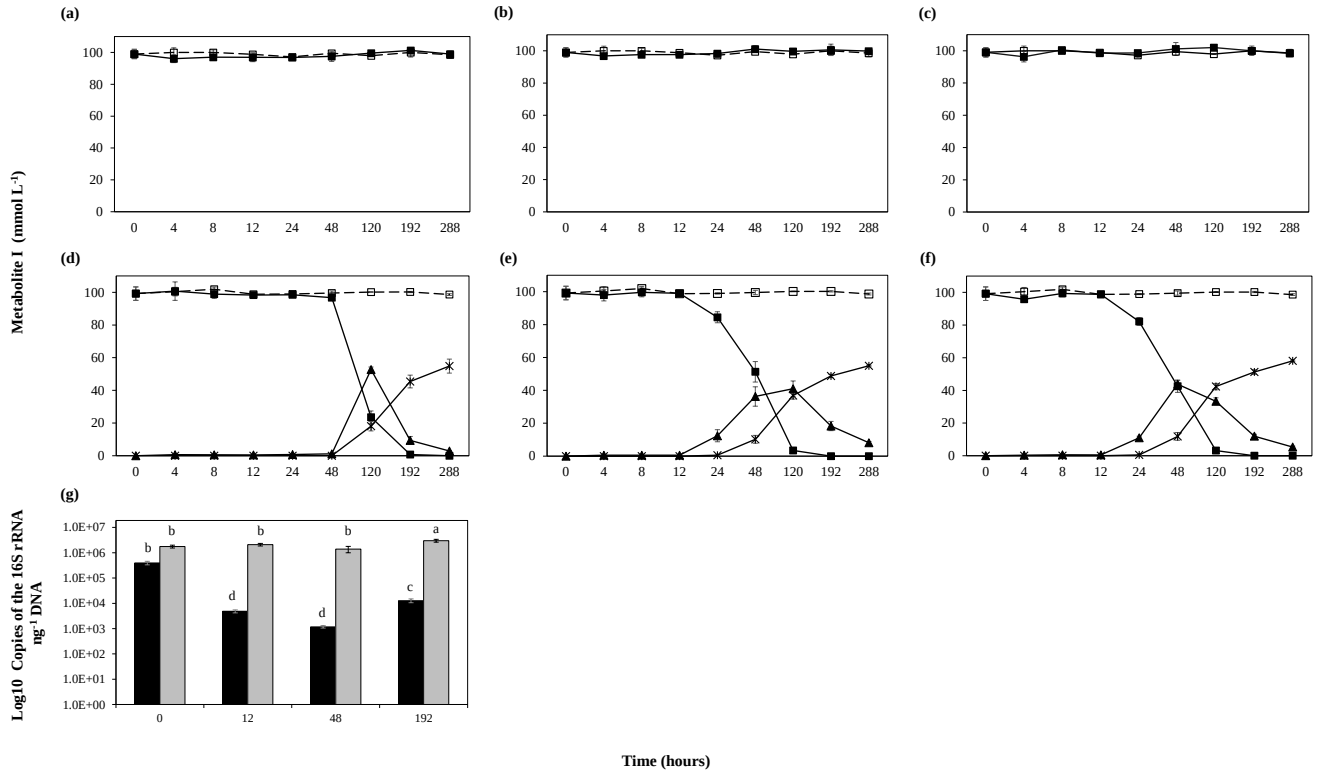



**Figure 3.3.** Degradation of 3,5-dichlorophenyl-carboxamide (metabolite I) (■) and formation of metabolite II (▲) and 3,5-DCA (∗) in MSM (a, b, c) and LB (d, e, f) inoculated with strains *Arthrobacter* C1 (a, d) *Achromobacter* C2 (b, e) and their combination (c, f). The degradation of metabolite I in non-inoculated controls is also shown (□, dotted lines).The growth of strains *Arthrobacter* sp. C1 (black bar) and *Achromobacter* sp. C2 (grey bar) co-inoculated in LB (g) were determined by q-PCR quantification of their 16S rRNA genes. Bars followed by the same letter are not statistical different at the 5% level. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

### 3.3.3. The degradation and metabolism of metabolite II

The degradation of metabolite II in both tested media was best described by the SFO kinetic model. Both strains and their combination were able to transform metabolite II to 3,5-DCA, but at variable rates depending on the strain and the media tested (**Figure 3.4**). Thus, stain C1 and its combination with C2 degraded metabolite II at a significantly faster rate in MSM ($DT_{50s}$ of 2.9 and 6.5 h respectively) compared to LB (135.6 h and 46.24 h). On the other hand, strain C2 was able to fully transform metabolite II only in LB ($DT_{50}$= 50.2 h), and only partial degradation of metabolite II was achieved in MSM ($DT_{50}$ = 363.9 h). No appreciable abiotic degradation of metabolite II was observed.

When bacterial growth was followed, different responses by the two strains in the different media were observed. Thus strain C1 showed a significant decrease ($p<0.05$) in its abundance in both MSM and LB until 48 h post inoculation followed by a further decrease at 192 h in MSM (**Figure 3.4g**) in contrast to LB where a recovery of its population was observed (**Figure 3.4h**). Regarding strain C2 a similar growth pattern as strain C1 was evident in MSM where its populations significantly decreased ($p<0.05$) at 12 h and remained stable from then onwards (**Figure 3.4g**). A more variable pattern was observed in LB where its population significantly increased ($p<0.05$) at 12 h, declined at 48 h and reverted to levels similar to the staring population at 192 h (**Figure 3.4h**).

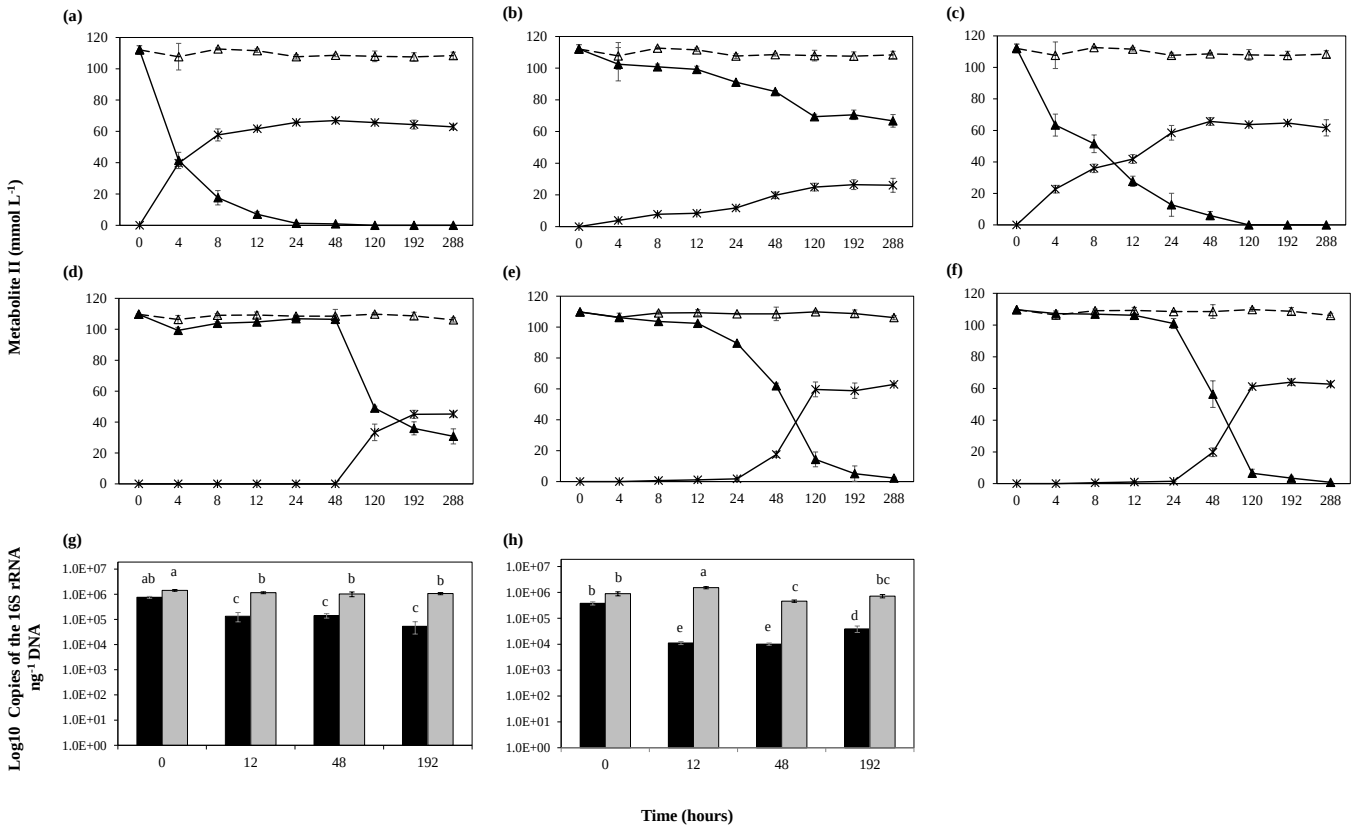



**Figure 3.4.** Degradation of 3,5-dichlorophenyurea acetate (metabolite II) (▲) and formation of 3,5-DCA (∗) in MSM (a, b, c) and LB (d, e, f) inoculated with strains *Arthrobacter* C1 (a, d) *Achromobacter* C2 (b, e) and their combination (c, f). The degradation of metabolite II in non-inoculated controls is also shown (□, dotted lines).The growth of strains *Arthrobacter* sp. C1 (black bar) and *Achromobacter* sp. C2 (grey bar) co-inoculated in MSM (g) and LB (h) determined by q-PCR quantification of their 16S rRNA genes. Bars followed by the same letter are not statistical different at the 5% level. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

### 3.3.4. The degradation and metabolism of other pesticides by *Arthrobacter* strain C1

The capacity of the main iprodione-degrading strain C1 to transform other pesticides carrying similar chemical moieties with iprodione was investigated in MSM. Strain C1 was not able to transform any of the phenylurea herbicides tested diuron, isoproturon, linuron and it also failed to degrade the dicarboxamide procymidone (data not shown). In contrast, strain C1 managed to degrade partially vinclozolin and propanil with their degradation patterns best described by the SFO kinetic model. Strain C1 significantly accelerated the degradation of vinclozolin ($DT_{50}$=106.3 h) compared to its abiotic control ($DT_{50}$=812.6 h) where 35% degradation of vinclozolin was evident at the end of the incubation (**Figure 3.5a**). The degradation of vinclozolin was accompanied with the formation and accumulation of 3,5-DCA with significantly higher amounts ($p<0.05$) observed in the inoculated compared to the non-inoculated cultures (>25 mmol $L^{-1}$ compared to 8 mmol $L^{-1}$). Vinclozolin hydrolysis was not accompanied by a significant bacterial growth ($p>0.05$) which increased from 2.5 x $10^8$ to 2.9 x $10^9$ CFU $mL^{-1}$.

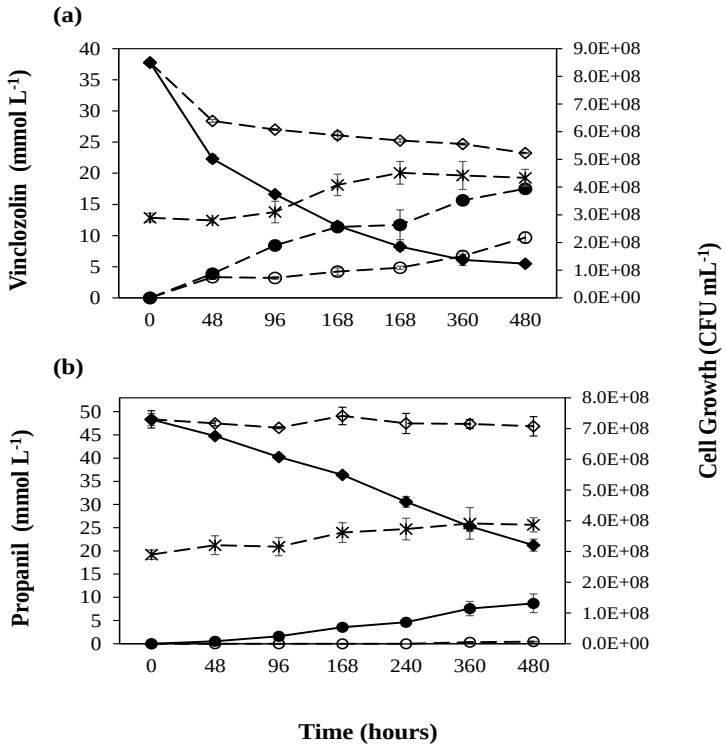



**Figure 3.5.** The degradation of (a) vinclozolin and (b) propanil (◆) and the formation of 3,5-DCA and 3,4-DCA (●) respectively in MSM inoculated with *Arthrobacter* sp. strain C1 and in (open symbols, dotted lines) non inoculated controls. Bacterial growth (∗) during degradation of the two pesticides is also shown. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

Strain C1 was also able to partially degrade propanil ($DT_{50}$= 389.9 h) with the formation and accumulation of small amounts of 3,4-DCA (**Figure 3.5b**). The degradation of propanil was not followed by a significant increase ($p>0.05$) in bacterial population, although an increasing trend was observed.

## 3.4. Discussion

Two strains previously isolated from an iprodione-degrading consortium (Campos *et al*., 2015) were tested for their ability to transform iprodione and its intermediate metabolites aiming to fully elucidate the metabolic pathway of the fungicide and the role of each strain on the successive steps of the metabolic pathway. Both strains metabolized iprodione via initial hydrolysis to isopropylamine and 3,5-dichlorophenyl-carboxamide (metabolite I) which was then transformed to 3,5-dichlorophenylurea acetate (metabolite II) and this in turn to 3,5-DCA which accumulated in the media (**Figure 3.6**). This pathway is similar to the one first described by Athiel *et al*. (1995) and further verified by Mercadier *et al*. (1997) however the nature of each transformation step was not explored in those studies.

The strains tested showed variable catabolic properties, nutritional needs and degradation rates. *Arthrobacter* strain C1 was the only of the two strains that was able to utilize iprodione and metabolite II as C and N source but failed to do so with metabolite I which was only degraded in the presence of extra C and N sources (LB). On the other hand, *Achromobacter* strain C2 was able to degrade iprodione and its metabolites only in the presence of extra C and N sources (LB). The capacity of both strains to only co-metabolize metabolite I contrasts findings by Mercadier *et al*. (1997) who suggested that this transformation step is mostly abiotic and it can only be accelerated by microbial mediation. However their study was performed at minimal media with pH of 6.5 which might have favored abiotic degradation of metabolite I compared to our study where the pH of all media was adjusted to 5.0 to minimize abiotic hydrolysis. The vulnerability of all dicarboxamides to abiotic hydrolysis under neutral to alkaline conditions is well documented (Szeto *et al*. 1989; Villedieu *et al* 1994). In contrast to the co-metabolic transformation of metabolite I, the following transformation step (transformation of metabolite II to 3,5-DCA) was an active catabolic process for *Arthrobacter* strain C1 which utilized metabolite II as C and N source. A particular observation was the significant reduction in the capacity of

*Arthrobacter* strain C1 to transform metabolite II in nutrient rich medium (LB). This is in line with the slower degradation of iprodione by strain C1 in LB compared to MSM and it could be attributed to the oligotrophic character of *Arthrobacter* strains (Poindexter 1981) which enables them to survive under extreme nutrient limitation (Cacciari and Lippi 1987).

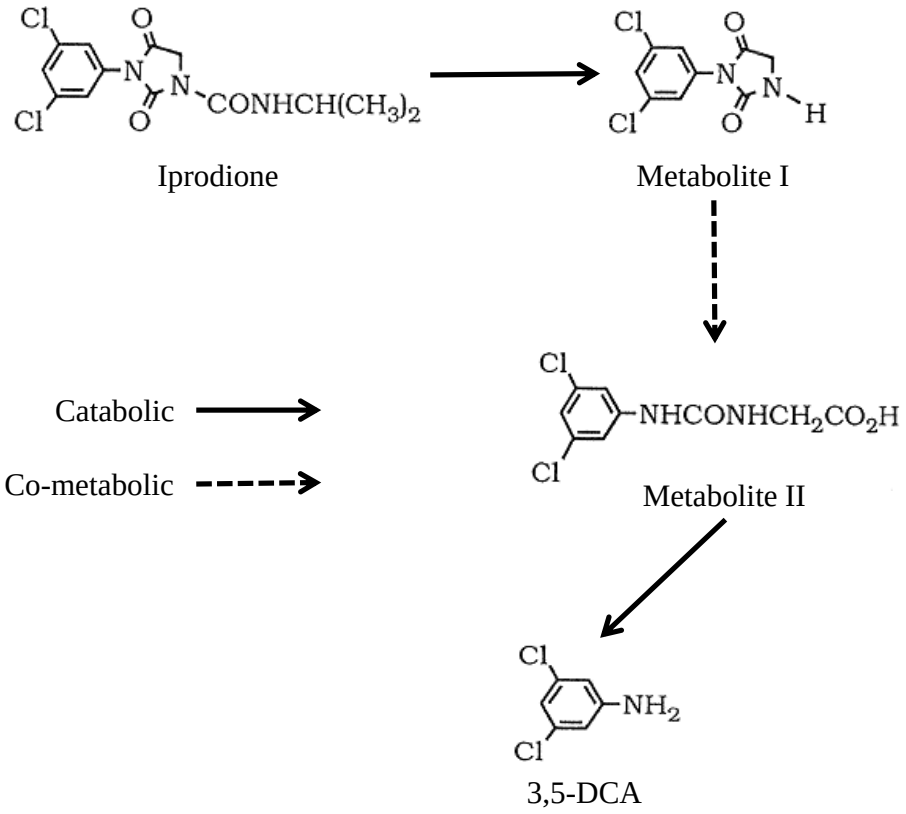



**Figure 3.6**. The metabolic pathway of iprodione by *Arthrobacter* strain C1. The nature of the microbial process involved (catabolic vs co-metabolic) in each step is demonstrated by different arrows.

The capacity of *Arthrobacter* strain C1 to utilize iprodione and metabolite II as the sole C and N source did not concur with an increase in their population (when co-inoculated with strain C2). This is in accordance with previous studies with iprodione-degrading which did not observe a significant bacterial growth despite the rapid consumption of iprodione (Athiel *et al*. 1995, Mercadier *et al*. 1997; Golovleva *et al*. 1991). Mercadier *et al*. (1997) attributed this lack of growth response to the release of low amounts of energy by the metabolism of iprodione which could not support

substantial bacterial growth. On the other hand, in LB supplemented with iprodione or metabolite I the maximum formation of metabolite I coincided with a drastic decline in the population of *Arthrobacter* strain C1 followed by a late recovery of its population when metabolite I had been transformed. This growth response of *Arthrobacter* strain C1 provides first evidence for a toxicity effect of metabolite I to the main iprodione degrading strain. In line with this hypothesis is the lack of late recovery of the population of the *Arthrobacter* strain C1 in MSM supplemented with iprodione where metabolite I accumulated. Overall, our data suggest that the transformation of metabolite I by *Arthrobacter* strain C1 is a co-metabolic process which is driven by the toxicity of the specific intermediate metabolite to strain C1. Recent proteogenomic analysis of pesticide-degrading bacteria showed that exposure of those bacteria to high pesticide concentrations trigger an intracellular toxicity response to bacterial cells (Breugelmans *et al*. 2010; Bers *et al*. 2011) suggesting that pesticide degradation by bacteria could be also a detoxification mechanism especially during co-metabolic processes where there is no energy gain. In contrast *Achromobacter* strain C2 did not show any growth response in MSM in accordance with its general limited capacity to actively degrade iprodione and its intermediates. In addition, no clear correlation between the growths of *Achromobacter* strain C2 in LB and degradation kinetics were observed stressing the secondary role of this strain in the degradation of iprodione.

Within the frame of elucidation of the metabolic capacities of the two isolates we investigated the degradation capacity of the main degrading strain, *Arthrobacter* C1, against pesticides of the same chemical group. *Arthrobacter* strain C1 was able to degrade only vinclozolin but failed to degrade the other dicarboxamide fungicide tested, procymidone. The degradation of vinclozolin coincided with the formation of 3,5-DCA which is in accordance with previous studies with vinclozolin-degrading *Pseudomonas* sp. (Cain and Mitchell, 1996), *Corynebacterium* sp., *Bacillus* sp. (Golovleva *et al*., 1991) and *Rhodococcus* sp. (Lee *et al*., 2008). The detection of appreciable amounts of 3,5-DCA in the non-inoculated controls suggests that abiotic process also contributed to the hydrolysis of vinclozolin, although overall the hydrolysis process was mainly microbially mediated. Previous soil studies have reported cross adaptation between iprodione and vinclozolin in iprodione-treated soils (Walker 1987; Cain and Mitchel 1996), however this is the first report of iprodione-degrading bacterium which is able to degrade vinclozolin. Dicarboxamide fungicides share a common 3,5-

dichlorophenylamide moiety linked to a substituted five-member heterocyclic moiety which varies between the different dicarboxamides: vinclozolin carries an oxazolidine ring, iprodione an imidazole ring, and procymidone a pyrole ring. Electrolytic studies showed that the oxazolidine and imidazole rings of vinclozolin and iprodione respectively were more prone to fusion resulting in the production of 3,5-DCA, compared to the heterocyclic moiety of procymidone which was stable (Pospíšil *et al*., 1999). This is in line with the limited capacity of the strain C1 to degrade vinclozolin but not procymidone.

Strain C1 was also able to partially degrade propanil via hydrolysis of the carboxamide bond which resulted in the formation of small amounts of 3,4-DCA. This metabolic pathway has been reported before in various propanil-degrading bacteria although in those studies the degradation rates were substantially higher (Hirase and Matsunaka, 1991; Zhang *et al*., 2011; Herrera-González *et al*., 2013). Hydrolysis of propanil by strain C1 was not followed by appreciable growth which is in agreement with the general lack of growth response also during hydrolysis of iprodione and vinclozolin. The limited capacity of strain C1 to hydrolyze the carboxamide bond of the urea moiety of diuron, linuron and isoproturon is in contrast to its capacity to actively hydrolyze the carboxamide bond of the urea moiety of metabolite II. Previous studies by Cullington *et al*. (1999) showed that the size or the nature of the urea N′-substituents were the main factors determining the capacity of an *Arthrobacter* strain to degrade different phenylureas. Thus it is possible that the higher affinity of *Arthrobacter* strain C1 to degrade metabolite II and propanil could be attributed to their similar N-substituents, acetate and propionate respectively, compared to the dimethyl or methyl-methoxy substituents in the phenylureas tested which might not facilitate the activity of the carboxamidases carried by *Arthrobacter* strain C1.

## 3.5. Conclusion

Metabolism of iprodione by two bacterial strains, an *Arthrobacter* C1 and an *Achromobacter* C2, purified from an iprodione-degrading consortium was investigated under variable nutritional conditions. The two bacteria exhibited variable nutritional requirements but employed the same metabolic pathway: an initial hydrolysis of iprodione to isopropylamine (only formed under selective growth conditions) and metabolite I, the latter was transformed to the intermediate metabolite II before being

hydrolyzed to 3,5-DCA. *Arthrobacter* strain C1 was the only of the two strains which was able to utilize iprodione and its intermediate metabolite II as C and N sources whereas it could only co-metabolically transform metabolite I. The latter step appeared to be a detoxification rather than a growth-driven transformation step. In contrast, *Achromobacter* strain C2 was only able to co-metabolize iprodione and its intermediates. Overall our data suggest that the microbial transformation of metabolite I to metabolite II appears to be the rate-limiting step in the metabolic pathway of iprodione. The capacity of *Arthrobacter* sp. strain C1 to metabolize the structurally related dicarboxamide vinclozolin and the acetamide propanil but not procymidone and phenylureas suggest a specificity of the catabolic enzymes involved in the degradation of iprodione by *Arthrobacter* strain C1. Further genomic/transcriptomic analysis of strain C1 will shed light into the genetic elements involved in the different steps of the metabolic pathway of iprodione.

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# Chapter 4

## Approaches to the potential affecting factors for rhizodegradation of iprodione and 3,5-DCA by degrading strains

# Approaches to the potential affecting factors for rhizodegradation of iprodione and 3,5-DCA by degrading strains

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## Abstract

Current biorremedial advances to avoid pesticide soil contamination have been a focus of interest in recent years. Here, rhizodegradation that combines plant roots and bacterial degrading ability is a reliable option to attenuate impact of iprodione fungicide and its 3,5-dichloroaniline (3,5-DCA) metabolite. Therefore, characterization and evaluation of potential affecting factors are essential for a proper rhizodegradation. The objective of this study was to approach the potential affecting factors for rhizodegradation of iprodione and 3,5-DCA by degrading strains. Iprodione-degrading *Arthrobacter* sp. strain C1 and 3,5-DCA-degrading *Pseudomonas* sp. strain were evaluated for their growing and degrading abilities in a mineral salt medium supplemented with sodium citrate (MSM+SC) and soil extract medium (SEM), demonstrating that both strains were able to grow and degrade better in presence of a simple C source (MSM+SC) than in the complex nutrient pool (SEM). Due to the limited degrading rate of 3,5-DCA by *Pseudomonas* sp., the supplementation with different C and/or N sources was investigated. This experiment demonstrated that the rate was only improved when some C and N sources where supplied as assembly, which suggested a secondary utilization mechanism. Additionally, an antagonistic experiment using soil cultivable microorganisms, as well as a phytoxicity assay exposing *Lolium perenne* seeds at high levels of iprodione and 3,5-DCA demonstrated that soil microorganisms and contaminants had not negative effect on inoculum growth and seed germination, respectively.

Thus, this study showed that *Arthrobacter* sp. strain C1 had the best degrading performance, which together with the L. *perenne* seed resistance to the contaminants placed them as suitable subjects for future rhizodegrading experiments.

**Keywords:** Iprodione; 3,5-DCA; rhizodegradation; *Arthrobacter* sp. strain C1; 3,5-DCA-degrading *Pseudomonas* sp.

## 4.1. Introduction

Intensive agricultural activity in the last years has been the natural response to a growing worldwide demand for food. Thereby, supplementation of suitable agrochemicals such as pesticides, should be sufficient to meet this demand for a better productivity (Stokstad and Grullon, 2013). Nevertheless, although utilization of pesticides is beneficial for the activity, a large amount represents a potential toxic risk for human and environmental health (Hernández *et al*., 2013; Malhat *et al*., 2015).

Iprodione [3-(3,5-dichlorophenyl) N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] is a contact fungicide recommended for control of diverse phytopathogenic fungi affecting important crops such as tobacco, strawberry, pear, lettuce and vineyard (AFIPA; Grabke *et al*., 2014; Miñambres *et al*., 2009). It is a potential toxic and carcinogenic molecule to humans (USEPA, 1998) and moderately persistent in soil and water, being prone to be hydrolyzed in soils with neutral to alkaline pHs (Cayley and Hide, 1980; Walker, 1987). However, soil microorganisms are the main iprodione-degrading agents, being 3,5-dichloroaniline (3,5-DCA) the major produced metabolite (Vanni *et al*., 2000; Walker, 1987). This metabolite is considered the most persistent dichloroaniline isomer in soil (PBT Profiler) and potentially nephrotoxic to mammalians (Lo *et al*., 1990) and toxic for soil microorganisms (Rochkind *et al*., 1986). Considering the previous statements, the accumulation of iprodione and its metabolite residues in natural resources could result dangerous for human health and environmental quality and should be mitigated.

One economically feasible and eco-friendly technology aiming to avoid or mitigate the hazardous impact of pesticides as iprodione and its metabolites is bioremediation (Abhilash *et al*., 2012). Bioremediation is the acceleration of the natural metabolic process using

microorganisms, green plants or their enzymes to alter and breakdown organic contaminants as pesticides into harmless substances from contaminated sites (Glazer and Nikaido, 2007), being one of its most interesting *in situ* variants the denominated rhizodegradation. In it, the breakdown of the pesticides is achieved by competent soil microorganisms, usually bacteria, which are stimulated by presence of rhizosphere. Thereby, plant roots exudates as sugars, alcohols and organic acids can act as nutrient sources to enhance the microbial growth and activity (Dennis *et al*., 2010). Some of these root exudates (e.g. short chain organic acids) can also act as chemotactic signals to recruit beneficial microorganisms (Tan *et al*., 2013). Additionally, plant roots can improve the aeration of soil and facilitate the transport of water into the rhizosphere improving the microbial activity and as result, the dissipation of pesticides (Kudjo Dzantor, 2007). Nevertheless, even when rhizodegradation by itself could work effectively, sometimes unfavorable environmental conditions, high persistence of the target molecule and unable metabolism of indigenous microorganisms, can be restricting factors for a suitable rhizodegrading process (Gerhardt *et al*., 2009). In these kinds of situations, inoculation of rhizosphere with characterized and effective pesticide-degrading bacteria strains seems to be a suitable strategy to improve the dissipation, avoiding the accumulation of pesticides and their metabolites (El Fantroussi and Agathos, 2005).

Isolation of iprodione-degrading bacteria with potential uses as inoculum has been scarcely noticed. Therefore, Athiel *et al*., (1995) reported the isolation of an *Arthrobacter*-like strain bacteria able to degrade iprodione rapidly. After that, Mercadier *et al*., (1997) achieved the isolation of three *Pseudomonas* strains able to synergistically metabolize iprodione via formation of different intermediate metabolites with a higher accumulation of 3,5-DCA. In addition, little information about isolation of 3,5-DCA-degrading bacteria has been also reported, being *Rhodococcus* sp. (Lee *et al*., 2008), *Acinetobacter baylyi* (Hongsawat and Vangnai, 2011) and *Bacillus megaterium* (Yao *et al*., 2011) the current available strains. More recently, in a previous study aimed to isolate degrading bacteria to make up a suitable bioremedial inoculum, we reached the isolation of a bacterial consortium and a pure *Pseudomonas* sp. strain able to degrade iprodione and 3,5-DCA, respectively (Campos *et al*., 2015). Consortium purification resulted in the isolation of a pure *Arthrobacter* sp. strain C1 able to degrade iprodione completely until 3,5-DCA, whereas *Pseudomonas* sp. could degrade the metabolite partially. Therefore, due the sinergystic

performance to degrade iprodione completelly and its persistent metabolites, both strains seem to be suitable subjects to develop a degrading inoculum to combine it with the power of rhizodegradation. Nonetheless, previous information about biotic and abiotic affecting factors, such as competition with indigenous microorganisms, antagonistic interaction, nutrient utilization (Mrozik and Piotrowska-Seget, 2010; Ray, 2014), growth rates (van Veen *et al*., 1997), as well as plant resistance to the contaminants (Rausher, 2001), are fundamental aspects to evaluate the feasibility of rhizodegrading process incorporating our bacterial inoculum.

Thus, the main objective of the present study was to approach the potential affecting factors for the rhizodegradation of iprodione and 3,5-DCA by the respective degrading strains.

## 4.2. Material and methods

### 4.2.1. Chemicals

Iprodione (MW=330,17 g $mol^{-1}$) and 3,5-DCA (MW=162,02 g $mol^{-1}$) analytical standards (>97% purity) (Sigma-Aldrich, USA) were used for the preparation of 1,000 µg $mL^{-1}$ in stock solutions in acetonitrile and used for analytical purposes. Solutions of these standards in high quality DMSO (10,000 µg $mL^{-1}$) (Merck, Germany) were prepared, filter sterilized (0.22 µm-pore-size filter; Millipore) and used for contaminating the culture media.

### 4.2.2. Media

Mineral salt medium (MSM) was prepared according to Rousseaux *et al*., (2001) with slight modifications and had the following composition (g $L^{-1}$): $K_2HPO_4$ (1.6), $KH_2PO_4$ (0.4). $MgSO_4 \cdot 7H_20$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), salt stock solution (1 mL), vitamin stock solution (1mL) and $FeSO_4 \cdot 6H_2O$ stock solution (1 mL, 5.0 g $L^{-1}$). Salt stock solution contained (g $L^{-1}$): boric acid (2.0), $MnSO_4 \cdot H_20$ (1.8), $ZnSO_4$ (0.2), $CuSO_4$ (0.1), $Na_2MoO_4$ (0.25); vitamin stock solution was composed of (g $L^{-1}$): thiamin hydrochloride (0.1) and biotin (0.04). The vitamin and $FeSO_4 \cdot 6H_2O$ stock solutions were filter sterilized (0.22 µm

pore size; Millipore) and kept at 4°C. Basal mineral medíum was adjusted at pH 5.0 by addition of HCl, autoclaved and suplemented with the different stock solutions. When it was necessary, MSM was supplemented with 10 mL of sterile stock solutions (100 g $L^{-1}$) of sodium citrate (MSM+SC), glucose (MSM+Glu), succinate (MSM+Suc) or ammonium chloride (MSM+$NH_4$) as extra single C or N sources. Combinations of these stocks such as glucose + ammonium chloride (MSM+Glu+$NH_4$) and succinate + ammonium chloride (MSM+Suc+$NH_4$) as well as Luria Bertani (LB) broth were used as extra C and N sources. Additionally, a soil extract medium (SEM) was prepared according to Hamaki *et al*., (2005). Briefly, 500 g of an Andisol soil belonging to Freire series, were mixed with 1 L of 50 mM NaOH and incubated overnight at room temperature. The mixture was centrifuged for 15 min at 5,000 rpm, filtered twice through a Whatman N°1 filter and centrifuged again for 60 min at 15,000 rpm. The supernatant (extract) was adjusted at pH 5.0, filter sterilized and kept frozen (-20 °C) until used. Thus, before running the assays, extract was mixed with sterile deionized water at the same pH and volume (1:1 v/v). Solid LB and malt extract media (ME) were also prepared by addition of 20 g $L^{-1}$ agar (Difco$^{TM}$) before autoclaving. Finally, solid SEM contained proportional volume of extract and autoclaved deionized water with agar. All the liquid and solid media were adjusted at pH 5.0 to keep a suitable molecular stability of iprodione through the assays.

### 4.2.3. Growth curve and iprodione/3,5-DCA degradation by degrading strains

The cell growth rates of iprodione-degrading *Arthrobacter* sp. strain C1 and 3,5-DCA-degrading *Pseudomonas* sp. strain were evaluated through a growth curve in MSM+SC and SEM media. Thereby, sterile triplicate flasks containing MSM+SC and SEM amended with 30 mmol $L^{-1}$ iprodione were inoculated with *Arthrobacter* sp. strain C1 at a final concentration of 9.09 and 9.16 Log10 CFU $mL^{-1}$, respectively. Likewise, flasks containing MSM+SC and SEM amended with 60 mmol $L^{-1}$ 3,5-DCA were inoculated with *Pseudomonas* sp. at 10.03 and 10.49 Log10 CFU $mL^{-1}$, respectively. Each treatment was incubated by shaking (180 rpm) for 96 h at 28 °C with constant cell growth measurement. Thus, cell growth was determined spectrophotometrically at 600 nm, converted into unit forming colonies (CFU) per mL of sample medium (CFU $mL^{-1}$) and expressed as Log10

CFU $mL^{-1}$. Conversion was possible using a standard calibration curve according to Roehrs *et al*., (2012). Briefly, repeated dilutions were made by appropriate cell suspensions in NaCL 0.85% solution, from which 0.1 mL was spread wide on solid LB medium supplemented with 30 mmol $L^{-1}$ iprodione or 60 mmol $L^{-1}$ 3,5-DCA and incubated for 48 h at 28 °C. Then, cell colonies were counted and those dishes that did not have lower than 20 or higher than 300 colonies were chosen to construct the curve.

In addition, to obtain an approach about the degradation behavior in enriched media, MSM+SC and SEM were used as laboratory model of rhizosphere environment. Thereby, the above described *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. treatments as well as abiotic controls (uninoculated) were kept in incubation for 360 h under the same conditions. Thus, for every treatment and control, iprodione and 3,5-DCA degradation was constantly measured by HPLC-DAD method.

### 4.2.4. Enhancing degradation of 3,5-DCA by addition of extra C and/or nutrient sources

In order to improve the degrading rates of 3,5-DCA by *Pseudomonas* sp. strain, it was decided to test different C and/or N sources. Therefore, triplicate flask containing MSM supplemented with glucose (MSM+Glu), succinate (MSM+Suc) or ammonium chloride (MSM+$NH_4$) as extra single C or N sources, and glucose + ammonium chloride (MSM+Glu+$NH_4$), succinate + ammonium chloride (MSM+Suc+$NH_4$) and LB media as extra C and N source were amended with 120 mmol $L^{-1}$ 3,5-DCA and inoculated with *Pseudomonas* sp. (10.03 Log10 CFU $mL^{-1}$). Every treatment was incubated by shaking for 480 h at 28 °C. Thus, 3,5-DCA residues were measured immediately and 48, 120, 240, 360 and 480 h after inoculation by HPLC-DAD.

### 4.2.5. Antagonistic effect of indigenous microorganism in the degrading strains

In order to get a referential idea about a possible antagonistic or competitive response from the native microflora on the degrading inoculum for future rhizodegrading experiments, we carried out a simple and qualitative laboratory experiment. Briefly, a rhizosphere soil sample (1g) was diluted serially in sterile NaCl 0.85% solution ($10^1$ to $10^9$), spread on Petri

dishes (1 mL) containing solid LB and ME media respectively, and incubated for 4 d at 28 °C. Parallel, *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. glycerol stocks were inoculated in LB broth and grown by shaking for 2 d at 28°C. Thereby, bacteria were washed 3 times, re-suspended at $2.0x10^8$ CFU $mL^{-1}$ in sterile NaCl 0.85% and used to spread individually 0.1 mL on LB dishes Immediately, 2 cut agar discs (2 cm diameter) of grown soil bacteria (LB) and fungi (ME) from the suitable microbial abundance ($10^4$) were disposed touching their developed surfaces with those from the inoculated dishes and left for incubation for 8 d at 28 °C. Thus, appearance of clear halos or disruption in the *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. growths after 2, 4 and 8 d were interpreted as antagonistic or competitive effect.

### 4.2.6. Ryegrass (*Lolium perenne*) seed germination in contact with different levels of iprodione and 3,5-DCA

To select the most suitable *Lolium perenne* plant variety for future rhizospheric experiments, a seed tolerance test in presence of iprodione and 3,5-DCA was carried out under laboratory conditions. Thereby, seeds of Expo AR1 (diploid) and Banquet Endo5 (tetraploid) varieties were disinfected according to Phillips *et al*., (2012). Briefly, seeds were disinfected by washing 1 min with 95% ethanol, followed by a 10 min wash in 5.25% sodium hypochlorite water solution, rinsed 5 times with sterile deionized water and left to dry for 1 h at 30 °C. After that, 40 disinfected and dried seeds of each variety were deposited on clean Petri dishes containing filter paper N°2 moistened and contaminated at a concentration of 30, 90, 150 and 300 mmol $kg^{-1}$ iprodione and 60, 180, 300 and 600 mmol $kg^{-1}$ 3,5-DCA, individually. Controls were established as the same seed amount on filter paper moistened with sterile deionized water. Treatments and controls were kept under darkness for 7 d at 25 °C. Thus, the Relative Seed Germination (RSG %), Relative Root Elongation (RRE %) and Germination Index (GI %) were calculated according to Tiquia *et al*., (1996) and modified for the present study.

### 4.2.7. Pesticide residue analysis

Analyses were performed in a UFLC Shimadzu HPLC/DAD system equipped with a Shimadzu C18 VP-ODS (150 mm x 4.6 mm; Shimadzu, Japan). For pesticide residue extraction, culture media aliquots (500 µl) were removed, mixed with methanol HPLC grade (1 mL), centrifuged and analyzed. For SEM, aliquots (1 mL) were cleaned up through columns containing florisil® salt, eluted with acetonitrile, filtered (0.22 µm) and injected into the HPLC system. A mobile phase consisting in acetonitrile:water (70:30 v/v) as is described by Carlucci *et al*., (2005) was used to elute iprodione and 3,5-DCA at 4.8 and 3.8 min. All the analyses used a wavelength of 220 nm and a flow rate of 1 mL $min^{-1}$ with a temperature column of 30 °C. Instrument calibrations and quantifications were performed using pure reference standards. The recoveries of each analyzed molecules were >90%.

### 4.2.8. Statistical analysis

Data were analyzed by ANOVA parametric test and three samples were compared using analysis of means of Tukey HSD test. Differences were considered as significant at 0.05 levels. Statistical analyses were performed with SAS JMP statistical software version 11.0.0.

## 4.3. Results and discussion

### 4.3.1. Growth curve and iprodione/3,5-DCA degradation by degrading strains

The elaboration and comparison of kinetic growths in two different media containing extra C and N sources (MSM+SC and SEM) were useful to determine different bacterial growth parameters and their influence in iprodione degradation. Therefore, when *Arthrobacter* sp. strain C1 was incubated in MSM+SC and SEM plus iprodione, the *lag* phase was calculated close to 3.8 h and 6.5 h, followed by an exponential phase of 18.6 h and 22.0 h, respectively. Likewise, a generation time (G) of 8.23 h was reached in MSM+SC compared with the 11.93 h in SEM, which demonstrated that *Arthrobacter* sp. strain C1 had a higher and faster growth in MSM+SC than SEM (**Figure 4.1a**). On the other hand, our study demonstrated a rapid iprodione degradation in both media. However, significant statistical

differences ($p \leq 0.05$) showed that faster degradation occurred in MSM+SC than in SEM, with a total iprodione transformation in 8 and 12 h, respectively (**Figure 4.2a**). Additionally, scarce amounts of released 3,5-DCA were observed following a similar pattern demonstrated in previous studies using this strain (Campos *et al*., 2015).

Similarly to our results, studies carried out by Mercadier *et al*., (1997) reported that iprodione half-lives by a *Pseudomonas* sp. degrading strain were 6 h in MSM and 4 h in the same medium supplemented with $(NH_4)SO_4$ or glucose, indicating that supplementation with extra C and/or N sources was able to increase the degrading rate. Thereby, it suggested that presence of a simple carbon source (e.g. sodium citrate) could favor the degradation via fast bacterial growth, whereas presence of complex nutrient sources from SEM could delay the iprodione hydrolysis as result of a longer adaptation of bacteria to the complexity of this medium (Strong *et al*., 2002; Vangnai and Petchkroh, 2007). A similar phenomenon was observed when *Arthrobacter* sp. strain C1 was previously incubated in LB broth (Campos *et al*., 2015). However, differences in degrading rates between restrict and rich media were minimal as in our present work. Thereby, it has been reported that presence of alternative C and N sources in combination with pesticides, is not a limitation for a simultaneous degradation of both compounds (Dinkla and Janssen, 2003). Thus, although differences of degrading rates had statistical differences, the overview explained that presence of additional C and N sources did not affect notoriously the degradation of iprodione and was independent from the growth degree.

On the other hand, when *Pseudomonas* sp. was incubated in MSM+SC and SEM supplemented with 3,5-DCA, the *lag* phase was calculated near to 4.30 h and 8.51 h, followed by an exponential phase of 16.06 h and 15.07 h, respectively. The respective G of 3.62 h and 5.23 h for each medium demonstrated that the strain had a higher growth in MSM+SC than in SEM (**Figure 4.1b**). Likewise, 3,5-DCA degradation for *Pseudomonas* sp. had differences with their respective controls in both media. Thereby, after 360 h incubation the degradation was higher in MSM+SC than in SEM (**Figure 4.2b**). However, this longer incubation did not substantially improve its degrading rate compared with previous results (Campos *et al*., 2015). Therefore, as it happened with iprodione, the delay observed in SEM seemed to be related to the complexity of its nutrients as well as the presence of compounds such as

antibiotics and xylose that can act as catabolic repressors of degradation due to a simultaneous utilization (Biesterveld *et al*., 1994). Otherwise, it is possible that utilization of a more simple C source such as sodium citrate, could be the start for an increasing bacterial biomass able to enhance the secondary utilization of 3,5-DCA (Aranda *et al*., 2003; Rittmann, 1992). Likewise, it has been described that addition of simple extra C sources such as succinate, citrate and pyruvate, can increase the bacterial population and degradation of chloroanilines in 15%, 6% and 5%, respectively (Hongsawat and Vangnai, 2011). Likewise, Travkin and Golovleva, (2003), have reported that a 3,4-DCA-degrading P. *fluorescens* practically did not show a *lag* phase in presence of glucose, whereas it was extended with a low growth and 3,4-DCA degradation without glucose, which could be correlated with a suitable glucose-stimulation of enzymes involved in the aromatic ring cleavage. Otherwise, the sustained decreasing concentration of 3,5-DCA in SEM controls (as well as iprodione controls) could suggest that a complexation reaction with soil constituents of the medium, such as dissolved organic soil matter, could decrease the 3,5-DCA availability and mask the real bacterial degradation intensity (Droulia *et al*., 2011; Morales *et al*., 2013). Nonetheless, even when the degradation of 3,5-DCA was better in MSM+SC than in SEM, these values were not higher than 50% after 240 h, which showed the persistence of this molecule to be degraded by our strain.

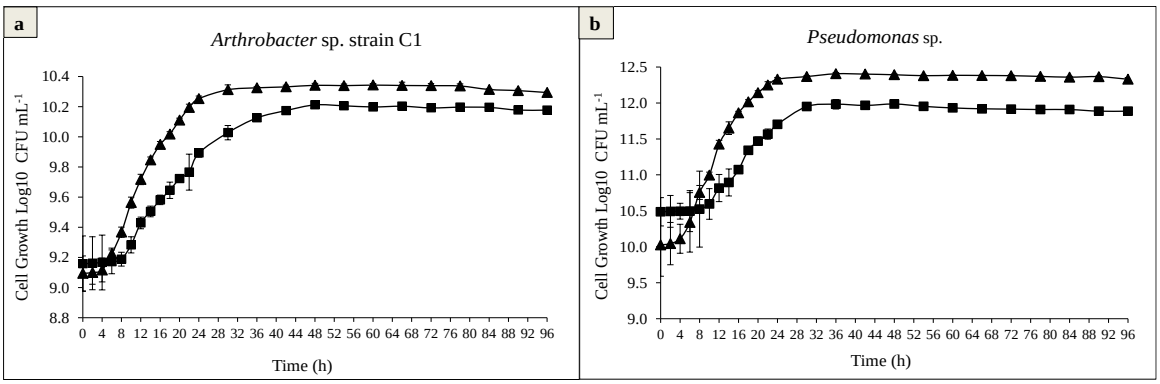


**Figure 4.1.** Growth curve of *Arthrobacter* sp. strain C1 (a) and *Pseudomonas* sp. (b) incubated in MSM+SC (▲) and SEM (■) adjusted at pH 5.0. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

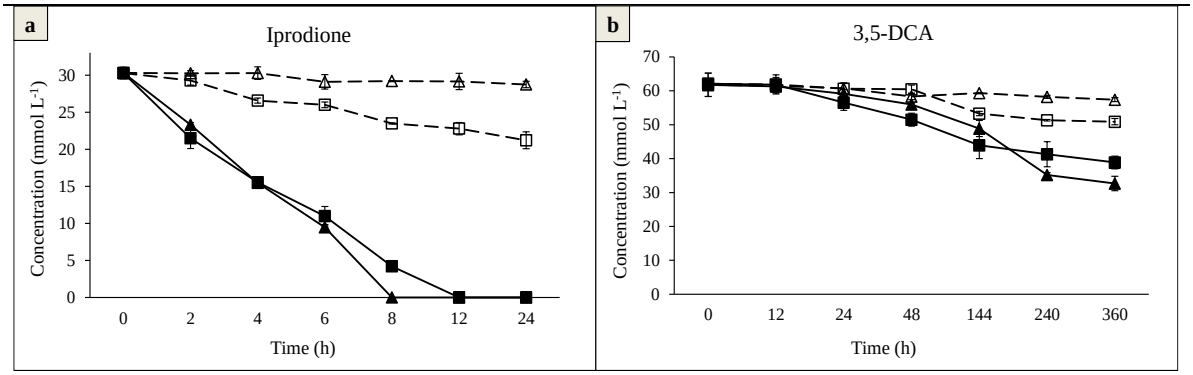


**Figure 4.2.** Degradation of iprodione by *Arthrobacter* sp. strain C1 (a), 3,5-DCA by *Pseudomonas* sp. (b) and uninoculated controls (open symbols, dotted lines) incubated in MSM+SC (▲) and SEM (■) adjusted at pH 5.0. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

### 4.3.2. Enhancing the degradation of 3,5-DCA by addition of extra C and/or nutrient sources

In order to improve the degradation of 3,5-DCA by *Pseudomonas* sp., an experiment utilizing extra simple C sources commonly founded in rhizosphere such as glucose and succinate (Dinkla and Janssen, 2003), ammonium chloride as alternative N source and LB broth as a rich nutrient medium, was investigated. Results showed significant statistical differences among bacterial treatments and abiotic controls only in treatments containing MSM supplemented with glucose and ammonium chloride (MSM+Glu+$NH_4$) (**Figure 4.3d**) and LB broth (**Figure 4.3f**) with a degradation of 50% and 60% respectively after 480 h. As it was mentioned above, addition of simple energy sources could result in an enhanced degradation of certain persistent organic molecules (Kanissery and Sims, 2011). Nevertheless, available information about enhancing degradation of chloroanilines by addition of extra C and N sources is controversial. In our study, we found that addition of glucose (**Figure 4.3a**), succinate (**Figure 4.3b**) and ammonium chloride (**Figure 4.3c**) by themselves or succinate plus ammonium chloride (**Figure 4.3e**) did not improve the degrading rates. Therefore, addition of glucose (Hongsawat and Vangnai, 2011) or ammonium chloride (Vangnai and Petchkroh, 2007) have been reported to induce reduction of degrading rates by extension in the *lag* period, which is coincident with our results. On the other hand, degradation of chloroaniline have been well related by addition of glucose

(Travkin and Golovleva, 2003) or succinate (Vangnai and Petchkroh, 2007), being opposite to the showed for us. However, regarding to the enhanced degradation in presence of glucose plus ammonium chloride, we could infer that C and N sources in assembly were necessaries to increase the protein synthesis for the growing biomass and improve the rate (Atlas and Philp, 2005), effect that by themselves were not able to emulate.

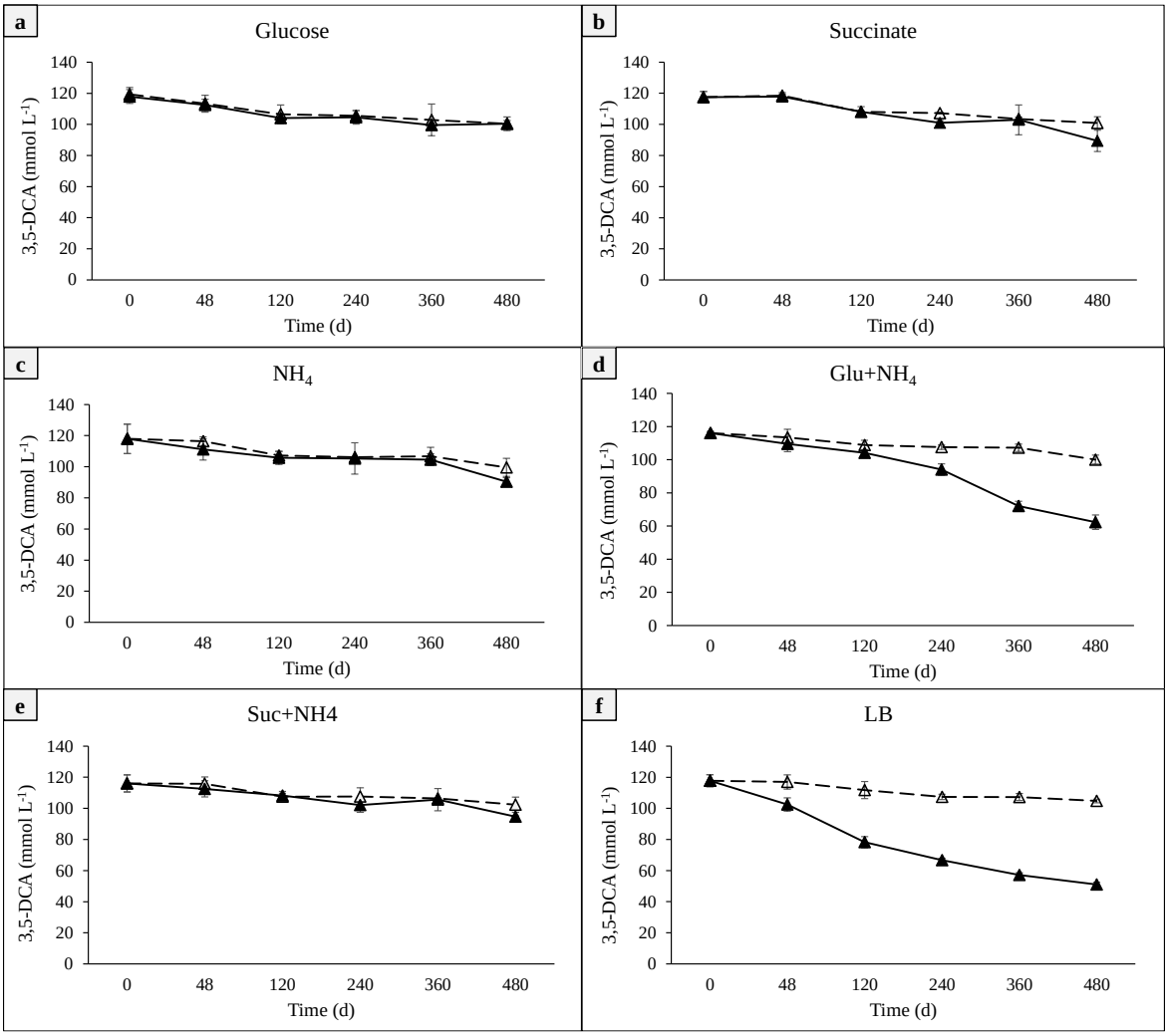


**Figure 4.3.** Degradation of 3,5-DCA by *Pseudomonas* sp. (▲) incubated in MSM supplemented with glucose (a), succinate (b), ammonium chloride (c), glucose + ammonium chloride (d), succinate + ammonium chloride (e) and LB broth (f) adjusted at pH 5.0. The degradation by uninoculated controls (∆, dotted lines) is also shown. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

In the present work we demonstrated that 3,5-DCA degradation was best improved when *Pseudomonas* sp. was incubated in LB broth. Therefore, it has been reported that addition of rich energy sources such as yeast extract can improve the degrading rates as higher concentration of this is added (Zhang *et al*., 2010). In these cases, nutrient sources seem to be the most suitable scenery to increase the cell number by a rapid way and with this, the degrading rate. Besides, it has been discussed that existence of particular molecules inside the nutrient pool can serve as inducers of genes involved in the degradation of the target compound (Hatzinger *et al*., 2001; Kim *et al*., 2008). Nonetheless, in our case the high resistance of 3,5-DCA to be degraded is well documented, being reported as the most persistent chloroaniline isomer due to the position of their halogenations (PBT Profiler). Consequently, the most suitable explanation for the degrading behavior by our strain is that it can poorly metabolize 3,5-DCA as primary substrate when it is the sole C and N source (Campos *et al*., 2015), whereas a secondary utilization mechanism can improve the degradation of this molecule only when a suitable amount of C and N sources is available to support its increasing bacterial biomass.

### 4.3.3. Antagonistic effect of indigenous microorganism on the degrading strains

Aiming at an easy and rapid approach to the possible antagonistic or competitive effect from cultivable microorganisms present in rhizosphere and our degrading inoculum, we carried out an in vitro experiment. In it, we expected to observe some changes in the growth aspect of the *Arthrobacter* sp. strain C1 (**Figure 4.4a**) and *Pseudomonas* sp. (**Figure 4.4b**) after 8 d of incubation by appearance of inhibitory halos. Nevertheless, inhibitory halos were not observed in presence of fungal and bacteria agar discs for both strains after this period. Therefore, there was only possible to note in some places that *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. did not grow, which was more attributed to the spreading technique than to an antagonistic or inhibitory effect, it because these places did not show colonization from the beginning (data not showed). However, it was possible to note in each dish that introducing bacterial agar discs resulted in an important colonization of the LB surface by indigenous soil bacteria. Likewise, fungal discs did not show important growth on the surface possible due to the nature of agar LB that is more suitable to bacteria than to fungi.

It is knows that competition with natural microbes for nutrients, antagonistic relationship, predation, growth rates and ability to use specific substrates are important limiting factors for the success of a degrading inoculum in environmental soils (Atlas and Philp, 2005). In this sense, competitive ability of bacteria in natural environments is difficult to study due to the varied metabolic activities of indigenous soil microorganisms (Duquenne *et al*., 1999). In the present experiment, we only tried to show an overview about possible affection for growth of inoculum, which was not detected. Nonetheless, activity and viability of introduced bacterial inoculum often show lower performances in contaminated sites compared with those observed under laboratory conditions, possibly due to diverse environmental stresses (Thompson *et al*., 2005). These facts are enough reasons by why our results have to be interpreted only as an approximation to the real scenario. However, it has been reported that inoculum safety can be improved by application into protected environments such as rhizosphere, where integrity and activity seem to be prolonged by longer period of time (de Boer *et al*., 2008; Lefevre *et al*., 2013), fact that might serve us for future rhizodegrading experiments.

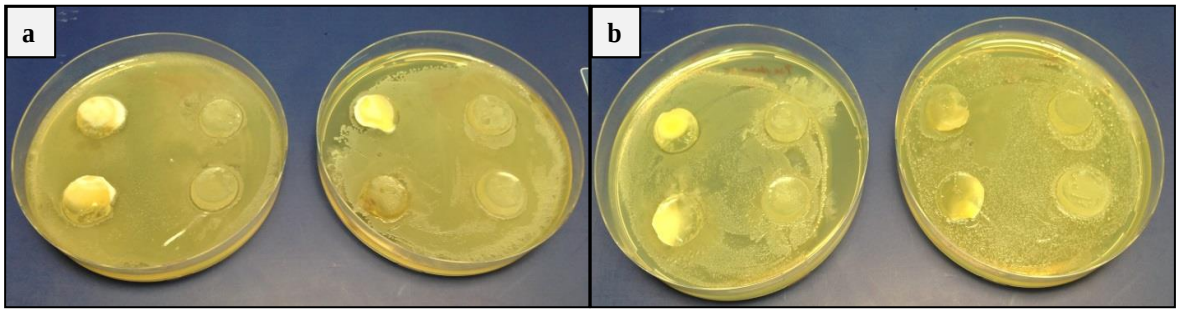


**Figure 4.4.** Antagonistic or inhibitory effect of cultivable indigenous soils bacteria and fungi on the growth of (**a**) *Arthrobacter* sp. strain C1 and (**b**) *Pseudomonas* sp. incubated in solid LB media. Effect was observed visually by apparition of clear inhibitory halos after 8 d incubation.

### 4.3.4. Ryegrass (*Lolium perenne*) seed germination in contact with different levels of iprodione and 3,5-DCA

According to previous works carried out on crop seeds, germination indexes (GI%) over 80% has been accepted as limit for disappearance of phytotoxicity in compost. Otherwise, values lower than 50% are indicators of a strong phytotoxicity (Zucconi *et al*., 1981a, 1981b). Based on this, our results demonstrated that iprodione concentration up to 90 mmol $kg^{-1}$ had GI values over 80% for both cultivars. Consequently, cultivars at 150 mmol $kg^{-1}$ as well as Expo AR1 at 300 mmol $kg^{-1}$ showed moderate phytotoxicity, whereas only Banquet Endo5 was noticed with a strong effect on the biggest iprodione concentration (**Figure 4.5a**). Scarce information about seed exposition and phytotoxic effect with fungicides such as iprodione has been reported. However, currently available information supports our encouraging results, even at higher iprodione concentrations. Thereby, it has been demonstrated that pre-treatment of crop seeds with iprodione to avoid infection by phytopathogenic fungi have no negative effect on seed germination, conversely improving germination and preventing the infection of susceptible seeds (Thomas and Sweetingham, 2003; Bankole and Joda, 2004; Rao *et al*., 2009). Likewise, Gange *et al*., (1992) tested the germination of several species of annual, perennial and semi-perennial forbs and grasses exposed to chlorpyrifos, dimethoate and iprodione, demonstrating that only chlorpyrifos and dimethoate had a significant effect, whereas iprodione had no negative effect. Therefore, as iprodione is a contact fungicide, it is possible that the diffusion into the seeds could be slower than other systematic pesticides, resulting in a less interference with enzymatic pathways of important hydrolases such as amylases, lipases, proteases and phosphatases (Bewley *et al*., 2013). Here it is known that the main phytotoxicity alteration by systemic pesticides is the reduction in the amylase activity with the consequent carbohydrate disturbances (Qian *et al*., 2009; Siddiqui and Ahmed, 2000). Thus, seed tolerance to pesticides has been related to low levels of storage carbohydrates in perennial species and a less reliance on amylase pathways (Gange *et al*., 1992).

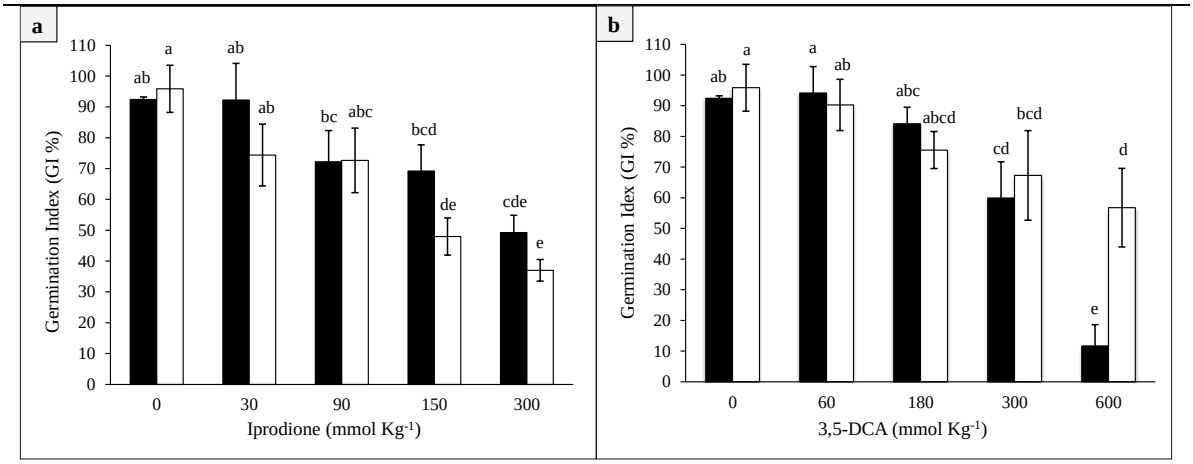


**Figure 4.5.** Germination Index (GI %) for seeds of L. *perenne* cultivar Expo Ar1 (black bars) and Banquet Endo5 (white bars), exposed at different iprodione (a) and 3,5-DCA (b) concentrations. Resulting values >80 % indicated no phytotoxicity, between 80-50 % represented an intermediate effect and <50 % constituted a stronger phytotoxicity. Each value is the mean of three replicates with error bars representing standard deviation of the mean.

Regarding to 3,5-DCA exposition, concentrations until 180 mmol $kg^{-1}$ for both cultivars and 300 mmol $kg^{-1}$ for Banquet Endo5 showed GI values over 80%. Intermediate GI values were for Expo AR1 at 300 mmol $kg^{-1}$ and Banquet Endo5 at 600 mmol $kg^{-1}$, being only Expo AR1 at this last concentration the treatment with a strong 3,5-DCA phytotoxicity effect (**Figure 4.5b**). There is no currently available existing information to compare. Nevertheless, seed germination of some plant species has been studied as reliable model of phytotoxicity for organic xenobiotics such as chloroanilines. Therefore, Hulzebos *et al*., (1993) noticed that lettuce seed (*Lactuca sativa*) germination in soil and nutrient solution was inversely proportional to the increasing concentration and lipophilicity/hydrophobicity degree of several chloroanilines. Likewise, Wang *et al*., (2001) suggested that chloroaniline hydrophobicity and electrophilicity were the main factors affecting germination and root elongation of cucumber (*Cucumis sativus*) seeds, which could be as result of a polar narcosis effect (Aruoja *et al*., 2014; Schultz *et al*., 1991).

Thus, due to the high resistance of the tested ryegrass seeds to iprodione and 3,5-DCA in the present experiment, we propose that both varieties could be suitable subjects to

incorporate in future rhizodegrading experiments and ensure a suitable performance for the removal of the contaminants.

## 4.4. Conclusions

Bacterial growth curve and degradation of iprodione/3,5-DCA demonstrated faster rates with short *lag* phases in MSM+SC than SEM, which was related with the simplicity of citrate as C source upon the complexity of SEM. At the same time, it was observed a total transformation of iprodione by *Arthrobacter* sp. strain C1, whereas degradation of 3,5-DCA by *Pseudomonas* sp. did not achieve more than 50%, fact that derived in new experiments in order to reach a suitable 3,5-DCA degradation. Therefore, the attempt to enhance the degradation of 3,5-DCA by *Pseudomonas* sp. incubated with different C and N sources showed slight improvements only in presence of glucose plus ammonium and LB broth, which demonstrated the limited metabolic ability of this strain to degrade the high resistant 3,5-DCA metabolite. Afterwards, the antagonistic and inhibitory experiment showed that soil bacteria and fungus did not affect the growth of *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. However, results reflected a restrictive spectra of the possible adverse effect by competition with the natural soil microflora being interpreted only as a reference. Testing the tolerance of two different varieties of *Lolium perenne* seeds to germinate in presence of high levels of iprodione and 3,5-DCA, it was determined that varieties had relatively high tolerance at every evaluated concentration, demonstrating that both varieties are suitable subjects to consider in next studies. Nevertheless, due to the excellent degrading performance of *Arthrobacter* sp. strain C1 and the limited ability of *Pseudomonas* sp. to degrade 3,5-DCA, it was decided only use the former strain to inoculate the grass cover of a biobed in future rhizodegrading experiments.

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# Chapter 5

## Enhanced iprodione removal by an *Arthrobacter* sp. degrading strain inoculated in the grass cover of a biobed

(Paper in preparation)

# Enhanced iprodione removal by an *Arthrobacter* sp. degrading strain inoculated in the grass cover of a biobed

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## Abstract

Biobeds are on-farm systems developed to avoid point contamination by pesticides such as iprodione and its metabolites, whose success is based on the degrading ability of microorganisms present in its biomixture. This biomixture is generally composed by peat, straw, soil and covered with a grass layer, being this a key component to improve pesticide removal by a process called rhizodegradation. Therefore, we aimed at enhancing iprodione removal by an iprodione-degrading *Arthrobacter* sp. strain C1 inoculated in the cover grass of a biobed. Biomixture microcosms planted and non-planted with ryegrass (*Lolium perenne*) were inoculated with *Arthrobacter* sp. strain C1 resulting in rhizosphere and non-rhizosphere inoculated treatments and rhizosphere and non-rhizosphere non-inoculated controls, which were contaminated with iprodione (90 mmol $kg^{-1}$) and kept for 49 days. Quadruplicate pots were periodically destroyed to measure enzymatic activities, root exudation of short-chain organic acids, iprodione and metabolite residues by HPLC and microbial abundances by qPCR. Results demonstrated that bacterial inoculation reduced at least twice iprodione half-lives ($DT_{50}$s), which was improved even more when plants were incorporated. Biomixture enzymatic activities showed an active biological role during early days without predominance for bacterial inoculum or plant presence. However, a high organic acid exudation demonstrated that plants were actively involved. The microbial analysis by qPCR showed that total fungi and total bacteria abundances decreased without any attribution to

plant or bacterial inoculation. Nevertheless, different bacterial taxa responded better under plant presence than the combination with bacterial inoculum, whereas no real changes were observed when inoculum was incorporated alone, revealing that iprodione removal was not determined by microbial abundances. Thus, our results show that inoculation with *Arthrobacter* sp. strain C1 strain and ryegrass incorporation are suitable alternatives to improve iprodione removal. Nevertheless, bacterial inoculation could need deeper studies to develop a feasible inoculum with reliable performance for on-farm biobeds.

**Keywords:** Iprodione; metabolites; *Arthrobacter* sp. strain C1; biomixture; rhizodegradation.

## 5.1. Introduction

Iprodione [3-(3,5-dichlorophenyl) N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] is a dicarboxamide fungicide commonly used in a wide range of crops to control fungal infestations by *Botrytis cinerea*, *Alternaria* sp., *Aspergillus* sp., *Penicillium* sp. and *Sclerotinia* sp., (Liu *et al*., 2009; Grabke *et al*., 2014; Hamada *et al*., 2011) and more currently, as an effective controlling agent of harmful root parasitic soil nematodes (Leysen and De Kerpel, 2012). Iprodione has been classified as probable carcinogen to humans (USEPA, 1998), and it is known to reduce testosterone production, hence delaying male rat pubertal development (Blystone *et al*., 2007). Its Koc of 400 mL $g^{-1}$ denote that iprodione is not strongly adsorbed onto soil particles (Strömqvist and Jarvis, 2005). As a result, iprodione is relatively mobile in the soil environment and its residues have been detected in drainage water from soils (Ludvigsen *et al*. 2004). However, due to the presence of the amino and aromatic group in the structure of iprodione and metabolites, their adsorptions can be relatively strong in soils with higher soil organic matter (SOM) content (Adrian *et al*., 1989; Morales *et al*., 2013).

Studies in soil and *in vitro* have shown that 3,5-DCA constitutes the major metabolite of iprodione and of other dicarboxamides (Vanni *et al*., 2000b). This metabolite is considered the most persistent dichloroaniline isomer in soil (PBT Profiler), toxic to soil microorganisms (Rochkind *et al*., 1986) and powerful nephrotoxic to rats (Lo *et al*., 1990). Considering all

the above statements, the accumulation of iprodione and 3,5-DCA residues in natural resources could be deleterious for human health and environmental quality and should be mitigated.

The close relation between plant roots and rhizosphere bacteria (rhizobacteria) with ability to metabolize pesticides can be an approach to improve the removal of iprodione and its metabolites by a bioremedial process called rhizodegradation (Gerhardt *et al*., 2009). Therefore, it is expected that rhizodegradation occurs strongly in places with a large exposition at high level of pesticides as on-farm biobed systems. Biobeds are biopurification systems (BPS) originated as a simple, low-cost and effective alternative to minimize environmental contamination from pesticide during its manipulation, especially when filling the spraying equipment on-farm (Castillo *et al*., 2008). A biobed is generally composed of a mixture of top soil, peat and straw (Swedish biomixture), and covered with a grass cover. Biomixture component offers an adequate pesticide sorption and effective degradation due to autochthone microorganisms present in soil. Straw stimulates extracellular ligninolytic enzyme production by microorganisms. Peat gives a potential sorption capacity, adequate moisture and a low pH adequate to enzyme activity (Castillo and Torstensson, 2007). Finally, grass cover helps to keep biomixture humidity, reveals pesticide spills due to point damage in grass (Torstensson and Castillo, 1997) and the most important feature is that it could improve pesticide removal for the retained pesticides in the first biobed layer by an active rhizodegrading process (Fogg *et al*., 2004b). Even though this phenomenon is probably successful, some environmental conditions can affect negatively the indigenous microbial activity (Gerhardt *et al*., 2009) and disfavor the rhizodegrading performance (Xu *et al*., 2009). In these scenarios, a suitable strategy could be the inoculation with known pesticide-degrading bacterial strains in the rhizosphere space improving the removal and avoiding the pesticide accumulation and their metabolites (El Fantroussi and Agathos, 2005).

In previous works, we reported the isolation and characterization of an effective iprodione-degrading *Arthrobacter* sp. strain C1 bacterium (Campos *et al*., 2015) with potential bioremedial use. Additionally, its metabolic pathway was investigated showing that under controlled conditions this strains is able to degrade iprodione at high amount of 3,5-dichlorophenylcarboxiamide (metabolite I) followed by a co-metabolic step to produce (3,5-

dichlorophenylurea) acetate (metabolite II) and 3,5-dichloroaniline (3,5-DCA), which was a desired aspect to avoid accumulation of more persistent metabolites as 3,5-DCA. Thus, due to the encouraging information about this strains, we decided to enhance the removal of iprodione by *Arthrobacter* sp. strain C1 inoculated in the cover grass of a biobed in this present work. The achievement of this objective can give important information about the removal process of iprodione and its metabolites in the cover grass of a biobed before and after the inoculation with our iprodione-degrading *Arthrobacter* sp. strain C1 and its use as suitable inoculum for future bioremedial application.

## 5.1. Material and methods

### 5.2.1. Chemicals

Formulated iprodione (Rovral® 50%WP) (MW = 330,17 g $mol^{-1}$) was diluted in distilled water at 15,144 mmol $L^{-1}$ and used for the contamination of biomixture. Pure iprodione and 3,5-DCA (MW = 162,02 g $mol^{-1}$) standards (≥ 97% purity) (Sigma-Aldrich, USA) and synthetized 3,5-dichlorophenylcarboxiamide (metabolite I) (MW = 245,06 g $mol^{-1}$) and (3,5-dichlorophenylurea) acetate (metabolite II) (MW = 262,07 g $mol^{-1}$), were used for the preparation of stock solutions in acetonitrile (ACN) and used for analytical purposes. Metabolites I and II were synthetized according to Athiel *et al.*, (1995) with slight modifications. Standards of oxalate, citrate, malate and succinate (≥ 96% purity) (Sigma-Aldrich, USA) were dissolved in distilled water acidified with *orto*-phosphoric acid (pH 2.6) at 1,000 μg $mL^{-1}$ and used for analysis.

### 5.2.2. Biomixture elaboration

For preparation of biomixture, an Andisol topsoil (0-20 cm) belonging to Freire series, wheat straw and commercial peat were used. Soil was dried and sieved (at 3 mm). Wheat straw was obtained from crop residues and cut in small pieces (≤ 2 cm). The components were strongly mixed at volumetric proportion (25:50:25 v/v) and the moisture content was adjusted at ± 60 % of water holding capacity (WHC) with distilled water. Biomixture was

disposed in a plastic content for maturation process in darkness at 20 ± 2 °C for 40 d before being used in the microcosm experiment. Some characteristics of biomixture are exposed in **Table 5.1**.

**Table 5.1.** Some chemical characteristics of biomixture used in this study.

| pH[a] | Electric conductivity[b] (µS $cm^{-1}$) | Potassium[c] (µg $g^{-1}$ soil dw) | Sodium[d] (µg $g^{-1}$ soil dw) | Phosphorous[e] (µg $g^{-1}$ soil dw) | Nitrogen[f] (%) | Organic Carbon[g] (%) | C/N[h] |
|---|---|---|---|---|---|---|---|
| 5.5 | 1,200 | 3,000 | 582 | 0.178 | 0.61 | 27.2 | 44.59 |

[a] Determined in soil: water suspension (1:4).

[b] Measured by a conductivity-meter (Consort C831).

[c, d] Extraction with ammonium citrate (1 M) at pH 7.0 and determined by atomic absorption spectrophotometry.

[e] Extracted by Bray-P1 method using 0.03 mol $L^{-1}NH_4F$ + 0.025 mol $L^{-1}$ HCl.as extractant.

[f] Extraction with KCl 2 mol $L^{-1}$ and determined by titration method

[g] Determined by wet combustion and reduced chromate colorimetric assay.

[h] Relation carbon/nitrogen.

### 5.2.3. Experimental set up and iprodione removal

To evaluate the removal of iprodione in rhizosphere and non-rhizosphere biomixture inoculated or not with a known iprodione-degrading bacterium (*Arthrobacter* sp. strain C1), a random microcosm experiment was carried out. A total of 112 pots (15x10x10 cm) filled with 60 g dw of biomixture were used to make up 2 different kinds of treatments and controls. Therefore, rhizosphere (B+P+C1) and non-rhizosphere (B+C1) treatments inoculated with *Arthrobacter* sp. strain C1 inoculum, and rhizosphere (B+P) and non-rhizosphere (B) controls non-inoculated were made. For B+P+C1 and B+P, pots were sown with ± 100 disinfected seeds of ryegrass (*Lolium perenne* var ExpoAR1) (Phillips *et al*., 2012) and placed in a growth chamber with 16 h photoperiod at 20-28 °C for 20 d (Louvel *et al*., 2011). Treatment and controls were kept with constant monitoring of humidity until bacterial inoculation. For the inoculum preparation, pure glycerol stock of *Arthrobacter* sp. strain C1

was inoculated into a sterile glass flask containing Luria Bertani (LB) broth amended with 60,6 mmol $L^{-1}$ iprodione and kept by shaking (180 rpm) at 28 °C for 2 d. After that, the bacterial pellet was obtained by centrifugation (6,000 rpm) of the flask content, washed 3 times with a sterile saline solution (NaCl 0.85%) and re-suspended at $8.3x10^7$ CFU $mL^{-1}$ with the same solution according to Campos *et al.*, (2015). From this, 6 mL were used to inoculate the closet superficial biomixture area to the plant roots in B+P+C1 and homogenously applied on the surface of B+C1 treatments, reaching a bacterial concentration of $8.3x10^6$ CFU $g^{-1}$. For B+P and B controls, saline solution without inoculum was added to keep the same humidity. Therefore, after 2 d inoculation, every treatment and control was contaminated with 6 mL of a formulated iprodione solution in water (909 mmol $L^{-1}$) to reach an iprodione concentration of 90 mmol $kg^{-1}$ dw. Then, quadruplicate pots were removed immediately after application, 7, 14, 21, 28, 35, and 49 d later. To obtain rhizosphere samples, B+P+C1 and B+P were uprooted, vigorously shaken and biomixture strongly adhered to plant roots was considered as rhizosphere. For B+C1 and B, the pot content was homogenized and considered as non-rhizosphere. In every case, plants and biomixture samples were used for immediate extraction of organic acids and enzymatic activity measurements, respectively. Enough amount of biomixture sample was stored at -20 °C until extraction and analysis of iprodione/metabolites residues and microbial quantification.

### 5.2.4. Root short-chain organic acids (OAs) extraction

Briefly, ryegrass roots samples were carefully washed 5 times with distilled water to clean their surface. Thereby, 5 g of clean roots were introduced in a glass beaker with 40 mL $CaCl_2$ 0.5 mM and kept in continuous aeration with a pump air for 1 h at room temperature. The supernatants (extracts) were filtered (0.22 µm-pore-size filter; Millipore) and frozen at -20 °C until analysis.

### 5.2.5. Enzymatic activities of biomixture

The β-glucosidase (GLU) as well as alkaline (AKP) and acid phosphatase (AP) activities were determined using 0.05 M *p*-nitrophenyl-β-D-glucopyranoside (PNG) dissolved in MUB buffer pH 6.0 for GLU (Eivazi and Tabatabai, 1988) and 0.05 M *p*-nitrophenyl phosphate

(PNPP) dissolved in MUB buffer pH 11 for AKP and pH 6.5 for AP (Tabatabai and Bremner, 1969) as substrates. Samples were incubated at 37 °C for 1 h and the release of *p*-nitrofenol (PNP) was measured spectrophotometrically at 420 nm.

Fluorescein diacetate (FDA) hydrolysis was measured according to Schnurer and Rosswall, (1982) with slight modifications. Briefly, 1 g dw of biomixture was incubated with 15 mL of buffer phosphate pH 7.6 and 0.2 mL of a FDA solution (2 mg $mL^{-1}$) as substrate at 30 °C for 20 min. The reaction was stopped by addition of chloroform:methanol (2:1), filtered (Whatman PS) and measured in a spectrophotometer at 490 nm.

Phenoloxidase activity was assessed using MBTH/DMAB method according to Castillo *et al*., (1994) with some modifications. Briefly, enzymes were extracted with sodium acetate buffer 0.05 M, pH 5.0 (Karanasios *et al*., 2010). Then, 300 µL of 6.6 mM DMAB, 100 µL of 1.4 mM MBTH and 30 µL of 20 mM MnSO4 were mixed with 1,560 µL of the filtered supernatant; the reaction started with the addition of 10 µL $H_2O_2$ 10 mM and measured at 590 nm. Finally, this activity represented the sum of peroxidase (LiP) and laccase (Lac) (Castillo and Torstensson, 2007).

### 5.2.6. HPLC analysis conditions

Iprodione, metabolite I, II and 3,5-DCA were extracted from biomixture following the method described by Wang *et al*., (2004) with slight modifications. Briefly, 5 g biomixture were mixed with 10 mL of ACN and shaken (200 rpm) for 1 h at 25 °C. Samples were centrifuged at 10,000 rpm for 10 min, filtered through PTFE membrane (0.22 pore size; Milipore) and directly analyzed by HPLC. For OAs, frozen samples were thawed and 10 mL were evaporated by a concentrator (speedvac®), re-suspended in 1 mL ultra-pure water pH 2.6 and injected into the HPLC system. The recoveries for iprodione, metabolite I, metabolite II, 3,5-DCA and OAs were >85%.

Samples were determined in a UFLC Shimadzu HPLC/DAD system equipped with a Shimadzu C18 VP-ODS (150 mm x 4.6 mm; Shimadzu, Japan). Regarding iprodione and 3,5-DCA, both were eluted using a mobile phase of acetonitrile:water (70:30 v/v) as described by Carlucci *et al*., (2005). For metabolite I and II, a mobile phase of

acetonitrile:water acidified at pH 2.6 with acetic acid (32:68 v/v) was necessary. Separation of oxalate, citrate, succinate and malate was reached using a methanol:water acidified at pH 2.6 with *orto*-phosphoric (5:95 v/v) as mobile phase. In all cases, a flow rate of 1 mL $min^{-1}$ was followed and the column temperature was at 30 °C. Instrument calibrations and quantifications were performed using pure reference standards.

### 5.2.7. DNA extraction

The DNA was extracted from samples (7, 21 and 35 d) with a MoBio Power Soil DNA isolation kit (Carls-bad, CA, USA) according to the manufacturer's protocols. A Homogenizer (minilys®) was used for biomixture lysis. The concentration and purity of the DNA was measured by emission of fluorescence with the Molecular Probes Qubit dsDNA BR assay kit using a Qubit® 2.0 Fluorometer (life technologies).

### 5.2.8. Quantification of microbial groups via qPCR

The abundance of total bacteria and different taxa were quantified using 16s rDNA qPCR assays, whereas the abundance of biomixture fungi was estimated via ITS qPCR assays (**Table 5.2**). qPCRs were carried out in 96-well racks at 15 µL reaction volume containing the SYBR green PCR MasterMix (Kapa SYBR® FAST qPCR Kit master mix (2X), KapaBiosystems, USA), 1 mM of each primer and 0.066-0.2 ng of DNA. Standard curves were obtained using serial dilutions of linearized plasmids containing the studied genes. The PCR efficiency for the different assays ranged between 78 and 98%. No-template controls were run for each quantitative PCR assay giving null or negligible values.

**Table 5.2**. Description of phyla and class-specific primers used for qPCR assays

| Target group | Primers | Amplicon Size (bp) | Annealing T (°C) | Reference |
|---|---|---|---|---|
| Total bacteria | 341_f/534_r | 194 | 60 | (Muyzer *et al*., 1993) |
| Actinobacteria | Actino235/Eub518 | 300 | 60 | (Fierer *et al*., 2005) |
| α-Proteobacteria | Eub338/Alf685 | 342 | 60 | (Fierer *et al*., 2005) |
| β-Proteobacteria | Eub338/Bet680 | 360 | 60 | (Fierer *et al*., 2005) |
| γ-Proteobacteria | Gamma395f/Gamma871r | 497 | 60 | (Mühling *et al*., 2008) |
| Firmicutes | Lgr353/Eub518 | 181 | 65 | (Fierer *et al*., 2005) |
| Fungi | ITS3F/ITS4R | 228 | 78 | (Mühling *et al*., 2008) |

### 5.2.9. Statistical analysis

Data were analysed by ANOVA parametric test and four samples were compared using analysis of means of Tukey HSD test. Differences were considered as significant at 0.05 levels. Statistical analyses were performed with SAS JMP statistical software version 11.0.0.

## 5.3. Results and discussion

### 5.3.1. Iprodione removal

The removal of iprodione in every treatment and control was best described by the single first order kinetic (SFO) model with the following equation:

$$C = C_0 e^{-kt}$$

where C = concentration of chemical at time t, $C_0$ = concentration of chemical present at time t = 0, k = removal rate. Therefore, significant statistical differences ($p < 0.05$) were obtained for iprodione removal, being this faster for B+P+C1 treatment ($DT_{50}$ = 3.35 d) than B+C1 ($DT_{50}$ = 4.59 d) treatment and B+P ($DT_{50}$ = 7.3 d) and ($DT_{50}$ = 9.5 d) controls (**Figure 5.1**). In general terms, iprodione removal for treatments and controls were relatively similar to previous works reporting high removal rates, where neutral to alkaline pH of biomixture was a relevant enhancing factor via abiotic hydrolysis of iprodione. Thereby, Karanasios *et al*.,

(2010) demonstrated that removal of iprodione was higher in biomixtures with pH ≥ 6.7 ($DT_{50}$ = 5 d) and those prepared with components at pH ≥ 6.57 ($DTs_{50}$ ≥ 9.8 d) (Karanasios *et al*., 2012). Similar results were demonstrated by Diez *et al*., (2013) where iprodione removals were higher in biochar based biomixtures at neutral to alkaline pHs than in wheat straw based biomixtures at acid pHs. Nonetheless, conversely to these studies, we demonstrated that iprodione removal was not related to abiotic hydrolysis, due to the acidic pHs present in our biomixture it was probably conducive to a better stability of the molecule (Walker *et al*., 1986; Walker, 1987). Instead, this enhanced iprodione removal could be indicative of an active microbial participation, fact that was supported by increasing levels of some biological parameters, aspect that will be deeply discussed in the following sections.

It is known that increasing the activity of indigenous pesticide-degrading microorganisms contained in the soil components of biomixture, is the key factor for a suitable BPS performance (Castillo *et al*., 2008). Thereby, to reach a maximum degrading rate, biomixtures should ensure previously a suitable proliferation of its microorganisms (Sniegowski *et al*., 2011), aspect that is not often possible to achieve (Fogg *et al*., 2004a). To overcome this problem, the biomixture inoculation with microorganisms containing catabolic pathways able to complete the mineralization of pesticide is suggested as an appropriate alternative. Therefore, von Wirén-Lehr *et al*., (2001) showed that inoculation with the white-rot fungi *Phanerochaete chrysosporium* removed 78 % of isoproturon after 28 d compared with the 76% in non-inoculated biomixture after 100 d. Bending *et al*., (2002) demonstrated that incorporation of *Trametes versicolor* removed 42.1 - 63.8 % of several pesticides after 42 d, including iprodione. Likewise, Ruiz-Hidalgo *et al*., (2014) demonstrated that carbofuran degradation in a biomixture inoculated with T. *versicolor* was higher than uninoculated control after 30 d. More recently, application of primed materials containing degrading microorganisms, such as soils with large history of exposure to pesticide, are subject of investigation (Sniegowski *et al*., 2011, 2012). However, studies evaluating pesticide removal by inoculation with pure or mixed exogenous bacteria in BPS have only been reported by Verhagen *et al*., (2013). These researchers demonstrated that inoculating a pure *Delftia acidovorans* strain and mixed bacterial cultures able to degrade chloropropham and 3-chloroaniline, the removal of the target chemicals were better than uninoculated control, suggesting a promising approach for future bacterial inoculations into BPS. Then, in

order to provide more information about the effectivity of degrading bacterial inoculum into biomixtures, we incorporated a previous characterized iprodione-degrading *Arthrobacter* sp. strain C1 in rhizosphere and non-rhizosphere biomixtures with potential future application for BPS.

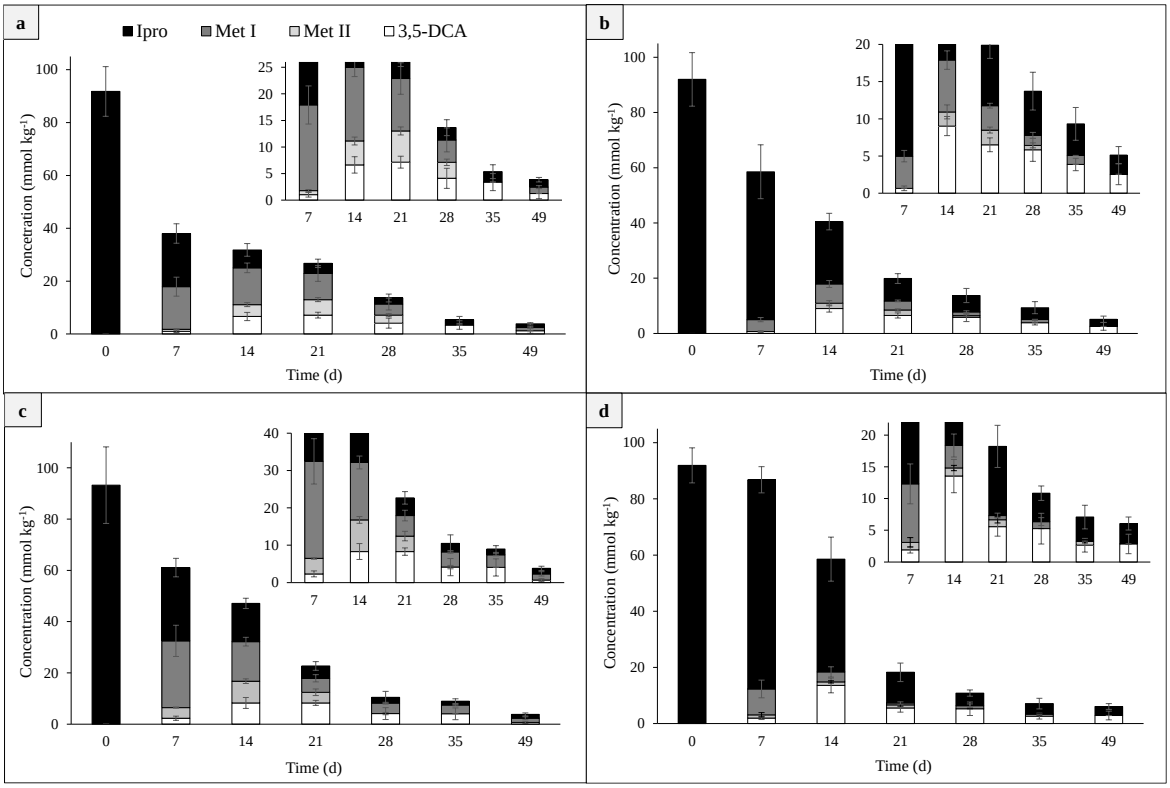


**Figure 5.1.** Removal of iprodione (ipro) and its metabolites (Met I, Met II and 3,5-DCA) by (a) rhizosphere inoculated treatment (B+P+C1), (b) rhizosphere non-inoculated control (B+P), (c) non-rhizosphere inoculated treatment (B+C1) and (d) non-rhizosphere non-inoculated control (B) incubated for 16 h of photoperiod d at 20-28 °C for 49 d. Small scale charts are included to improve the visualization of small concentrations. Each value is the mean of four replicates with error bars representing the standard deviation of the mean.

The comparison between rhizosphere (**Figure 5.1a**) and non-rhizosphere (**Figure 5.1c**) treatments did not show significant statistical differences for them $DT_{50}$ ($p < 0.05$) after 49 d of incubation. Nevertheless, contrasting treatments with the respective controls (**Figures**

**5.1b** and **d**) it was demonstrated that bacterial inoculation was critical to enhance their $DT_{50}$ twice at least. As it has been mentioned above, studies reporting enhancing removal of pesticide such as iprodione by inoculation with bacteria are scarce, being the nearest investigations those carried out in soil. Thereby, soil inoculation with iprodione-degrading bacterial strains was explored previously by Mercadier *et al*., (1996). These authors reached $DT_{50}$s twice less (30 - 60 d) than obtained for uninoculated soils inoculating non-adapted soils with an iprodione-degrading *Arthrobacter*-like strain. Comparing this, it is evident that our inoculated biomixture was, by far, faster than the results given by Mercadier *et al*., (1996), remarking that combination of biomixture and inoculation improved the removal process largely. On the other hand, similarities between both studies were possible to be established. Therefore, a large and transitory accumulation of metabolite I and, at lesser extent, metabolite II by B+P+C1 (**Figure 5.1a**) and B+C1 (**Figure 5.1c**) during the first days of assay was higher than the observed in controls. This behavior is a special aspect to be remarked, because the previous characterization of our *Arthrobacter* sp. strain C1 noticed that its metabolic pathway is producing mainly metabolite I from iprodione in culture media. However, presence of extra nutrient sources and additional metabolite degrading strains were factors to improve the co-metabolism of metabolite I with a transient metabolite II production until the final 3,5-DCA transformation (**Chapter 2** and **3**). Therefore, this fact could explain the negligible formation of metabolite II and the concomitant accumulation of 3,5-DCA, which could be favored by the nutrient pool presented in the biomixture as well as extra degrader microorganisms.

The evidence underlines that 3,5-DCA is the main produced metabolite after degradation of iprodione and other dicarboxiamide fungicides in soil (Vanni *et al*., 2000a, 2000b), being these relatively high resistant to degradation by bacteria (Hongsawat and Vangnai, 2011; Yao *et al*., 2011). Nevertheless, in our study this metabolite was only observed in a larger extent in B control (**Figure 5.1d**), in any case being a stoichiometric transformation of iprodione for this control or the remaining samples. Thereby, this effect appears to show that incorporation of *Arthrobacter* strain C1 and especially ryegrass root presence, improved the removal of this metabolite possibly by some additional rhizodegrading process such as plant absorption or immobilization (Gerhardt *et al*., 2009), which was evident by the negligible 3,5-DCA accumulation in B+P. However, evidences about this phenomenon were not

possible to be elucidated by these measurements, which will be discussed below in this report.

Finally, although the main removing factor for iprodione and its metabolites seemed to be by a microbial way, biochemical characteristics as a high organic carbon fraction present in our biomixture (**Table 5.1**) could play an important role stabilizing both molecules by absorption and complexation into the humic fraction. Then, it has been speculated that aromatic and amine groups present in the chemical structure of both contaminants are subject to be coupled by means of covalent bounds (Kong *et al*., 2013) to the humic acids present in soil colloids, which are able to inhibit the hydrolysis and increase their half-lives time (Morales *et al*., 2013) with a lower probability to be desorbed.

### 5.3.2. Enzymatic activities of biomixture

Significant statistical differences ($p < 0.05$) among treatments and controls were observed for activity of acid phosphatase (AP), alkaline phosphatase (AKP), β-glucosidase (GLU) and fluorescein diacetate hydrolysis (FDA) after 49 d experiment. In general terms, levels for AP in each case were carried out at higher degrees than those measured for AKP, which demonstrated that the acid pH present was a preponderant factor for a better activity of the acid isoenzyme. As results of this, different patterns for both enzymes were conducted through the experiment. Thereby, a first glance for AP it noted that B+P+C1 treatment and B+P control incremented their activities until 7 d with later normalization, in contrast with those for B+C1 treatment and B control that decreased gradually through time (**Figure 5.2a**) evidencing that plant incorporation resulted in an improvement on this parameter over the bacterial inoculation. On the other hand, AKP activity showed a clear decreasing rate for every treatment and control from 7 d, on which slowly started to normalize after this period. Here, differences among treatments and controls were not possible to establish and demonstrated unclear relation to bacterial inoculation or plant presence for these decreasing levels instead being more related with the application of iprodione (**Figure 5.2c**). Regarding this, the influence of pesticide, plant and microbial inoculum for phosphatases activities on BPS has been scarcely explored. Thereby, Tortella *et al*., (2013b) showed that addition of carbendazim fungicide had a strong negative effect on the AP activity during a short period

of time with a fast recovery, suggesting an adaptive response of biomixture microorganisms. In this study they also reported a lower AKP activity, possibly due to the acid biomixture pH, with a negative effect followed by normal levels. Additionally, the same group reported that AP activity was strongly affected by atrazine incorporation followed by a normalization after the first days, whereas AKP activity was evolving positively during all the experiment, which suggested a similar adapting response (Tortella *et al*., 2013a). However, adaptation of microbial communities related to phosphorous cycle could not be related to the improvement of pesticide degradation due to existence of contradictory studies reporting negative and positive results (Singh *et al*., 2002; Yun *et al*., 2006; Muñoz-Leoz *et al*., 2011; Srinivasulu *et al*., 2012; Karpouzas *et al*., 2014). On the other hand, the highest AP activity on 7 d showed in our study, could suggest an active root exudation as response for an increasing phosphorous solubilization (Tarafdar and Jungk, 1987) and a possible deficit condition of its element for the microorganisms (Hernandez *et al*., 2006; Van Moorleghem *et al*., 2013) under stressing conditions such as incorporation of high iprodione concentrations (Anzuay *et al*., 2015). Nevertheless, the utilization of phosphatase activity measurement as an index of microbial response to the pesticide stress results difficult to be used by itself (Perucci *et al*., 2000) due to variations in factors such as pesticide nature (Chishti *et al*., 2013) and physical, chemical or microbial properties of biomixture (Tortella *et al*., 2013a). Thus, even if this statement could be certain, the large AP activity could be related to an increasing microbial activity coincident with peaks for the remaining enzymes and the most active iprodione removal period, suggesting a role of AP in the process, which has not been elucidated, yet

As it was mentioned above, GLU activity reached its highest value on 7 d with a notorious increase for B+C1 treatment and B control. Nonetheless, a strong depletion was visualized for every treatment and control after this period (**Figure 5.2c**). β-glucosidase is a key enzyme involved in the carbon soil cycle, specifically in the degradation of cellulose and other β -1,4 glucans releasing sugar monomers (Ljungdahl and Eriksson, 1985). This enzyme is important for C recycling, hence it is expected that GLU should be higher in planted soil due to the constant root exudation (Kremer and Li, 2003; Acosta-Martínez *et al*., 2008). However, conversely to this statement, our results showed higher activity in non-rhizosphere biomixtures over rhizosphere, which appeared to express that plants were sensitive to iprodione and that inoculation did not exert a negative effect on this parameter (Lin *et al*.,

2011). Regarding this, pesticide contamination of biomixture has demonstrated to perform negative effects on GLU activity. Therefore, Tortella *et al*., (2013a) described that atrazine presence reduced the GLU activity with a later recovery in biomixture. Likewise, soils polluted with tebuconazole fungicide (Muñoz-Leoz *et al*., 2011) and nicosulfuron herbicide (Karpouzas *et al*., 2014) have demonstrated to be deleterious for GLU activity. Although our results highlighted a peak GLU activity on 7 d, the rest of the experiment was conducive at lower levels than in the initial days. Thus, we did not establish a conclusive relation between bacterial inoculation and plant root presence. Nevertheless, the increased GLU activity on 7 d was coincident with the strongest effect on remaining enzymes and iprodione removal establishing that possibly iprodione had the main role in this parameter.

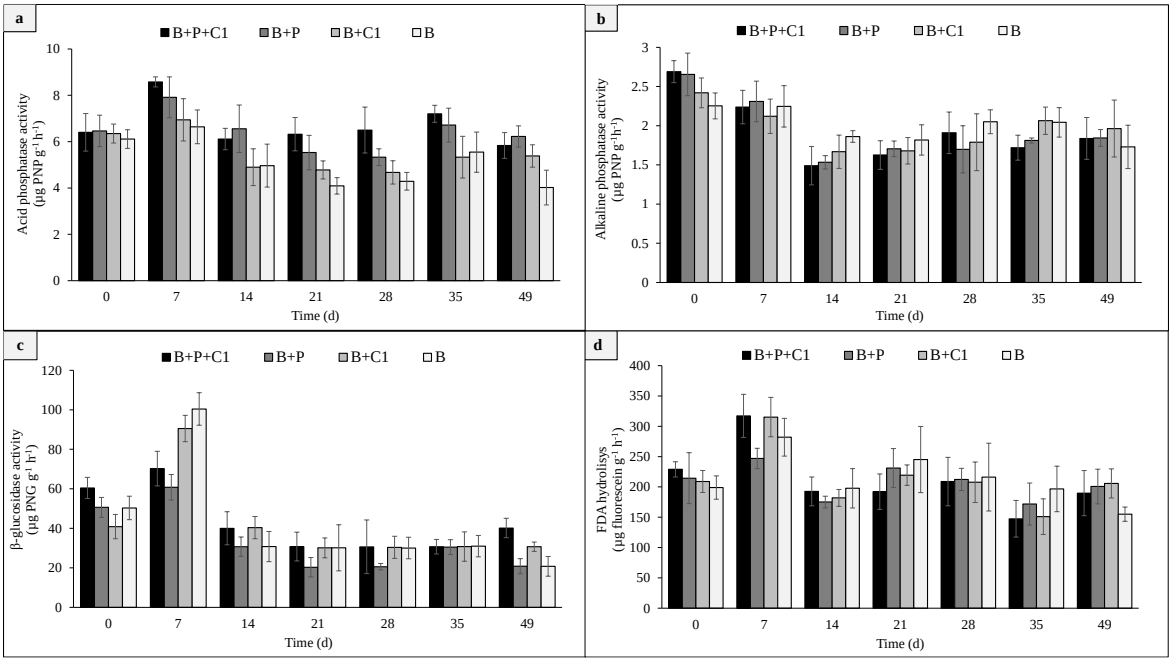


**Figure 5.2.** Enzymatic activity of acid phosphatase (a), alkaline phosphatase (b), β-glucosidase (c) and fluorescein diacetate hydrolysis (FDA) (d) in biomixture contaminated with iprodione. Rhizosphere inoculated treatment (B+P+C1), rhizosphere non-inoculated control (B+P), non-rhizosphere inoculated treatment (B+C1) and non-rhizosphere non-inoculated control (B) were incubated with 16 h of photoperiod at 20-28 °C for 49 d. Each value is the mean of four replicates with error bars representing the standard deviation of the mean.

In relation to FDA hydrolysis, increments on this parameter were possible to observe for every subject at 7 d, being this especially higher for inoculated treatments (**Figure 5.2.d**). Therefore, this behavior seemed to ratify that a high hydrolyzing activity was concomitant with the highest iprodione removal during the same period. It has been well described that iprodione and dicarboxiamide fungicide degradation is mainly carried out by hydrolases presented in degrading bacteria such as our *Arthrobacter* sp. strain C1 (Hashimoto *et al*., 2006) as well as in ubiquitous soil microorganisms (Acosta-Martínez and Tabatabai, 2001; Nannipieri *et al*., 2012). However, negative effects on the FDA activity by incorporation of iprodione have been reported previously in soils. Miñambres *et al*., (2009) showed that total FDA was reduced in presence of fungicide possibly due to a negative effect on the hydrolase enzymes involved in the process. Additionally, Podio *et al*., (2008) described that application of three broad spectrum fungicides had negative and positive effects on the biological soil activities, which have been supported by other researchers under similar conditions (Yun *et al*., 2006; Verdenelli *et al*., 2012; Gao *et al*., 2015). Therefore, even when FDA is considered as a suitable tool for estimation of early impact of pesticides on soil microbial activity (Perucci *et al*., 2000), its unspecific target needs to be correlated with extra parameters to clarify the outlook. In our study, we could correlate the increasing FDA activity with the remaining enzymes and the highest iprodione removal in a suitable way at the same time. After that, the normalization or slight detriment in FDA rates are indicating that in post days the biological activities were under light pressures or recovering until normal status previous iprodione contamination. However, not clear correlation between bacterial inoculation and root presence could be stablished to elucidate which aspect was the main factor during the process.

Finally, the search for phenoloxidase activity in any treatment or control had satisfactory results. Contrary to the broad literature about pesticide removal in BPS, we only found scarce activity at 0 d with null results for the remaining days (Castillo *et al*., 2008) (data not showed). However, extra studies have demonstrated a minimal contribution of these enzymes in pesticide degradation (Karanasios *et al*., 2010), possibly due to interferences derived from biomixture composition or the experiment conditions (Castillo *et al*., 1997).

### 5.3.3. Short-chain organic acids (OAs) exudation

In order to elucidate the plant participation during the removal of iprodione as well as the effect of bacterial inoculation to them, the exudation of different short-chain organic acids by ryegrass roots, referred only as organic acids (OAs) onwards, was analyzed. Thereby, exudation of oxalate, citrate, succinate and malate by uprooted plants from B+P+C1 and B+P showed different behaviors with statistical significant differences through the experiment ($p < 0.05$) (**Figure 5.3**). In general terms, it was possible to establish differences in the releasing pattern of the OAs during the experiment. At the first attempt, similar behaviors for oxalate (**Figure 5.3a**) and succinate (**Figure 5.3c**) released by treatment and control were observed with an increment during the first days (7 d and/or 14 d) and under lined depletion after that. Conversely, citrate (**Figure 5.3b**) and malate (**Figure 5.3d**) followed a constant increase from day 0 until the end of the experiment, excepting malate acid for B+P+C1, which was strongly depleted on 7 d. Differences between treatment and control were possible to be established as well. For example, B+P+C1 had higher amounts of oxalate, citrate and malate than B+P at 0 and 7d. However, afterwards these differences started to be similar or were invested such as the case of citrate. On the other hand, succinate showed high values for B+P compared with B+P+C1 during 0 and 7 d, which was by far the most released OA during this period.

It is known that stressing conditions such as contamination with pesticides are important stimuli to increase the root exudation in order to improve the microbial activities involved in the degradation of these organic contaminants (Ryan *et al*., 2001; Chaudhry *et al*., 2005). Among these root exudates, AOs have been demonstrated to participate as effective chemo-attract of specific beneficial bacteria at root surface (Compant *et al*., 2010; Tan *et al*., 2013) as well as to be able to increase the bacterial activity of degrading bacterial populations in rhizosphere (Muratova *et al*., 2009). In our study, we noted that some OAs were higher in the inoculated treatment than in the uninoculated control on the first days. However, the high exudation in both suggests that inoculation did not have a pronounced effect and could be more related to the application of iprodione on 0 d. Here, the increment at the levels on early days was coincident with peaked enzymatic activities and active iprodione removal demonstrating that plants played an active role. Therefore, it has been raised that root exudation of citrate, malate, malonate, fumarate and oxalate can participate in the chelation

of polyvalent cations improving the bioavailability of organic contaminants by dissolution of SOM complex, where these molecules are retained, and improve their degradation via microbial (White *et al*., 2003; Ling *et al*., 2009) or physical-chemical process prevailing in the matrix (Ou *et al*., 2015). This fact has been ratified by rhizodegrading studies for combined pesticides in biomixture rhizosphere (Diez *et al*., 2015) as well as for dichlorodiphenyltrichloroethane (DDT) (White *et al*., 2003), hexachlorocyclohexane (HCH) (Kidd *et al*., 2008) and polycyclic aromatic hydrocarbons (PAHs) (Ouvrard *et al*., 2006; Muratova *et al*., 2009) in soil rhizosphere, where exudation or supply of OAs improved the SOM dissolution or the microbial activity in rhizosphere.

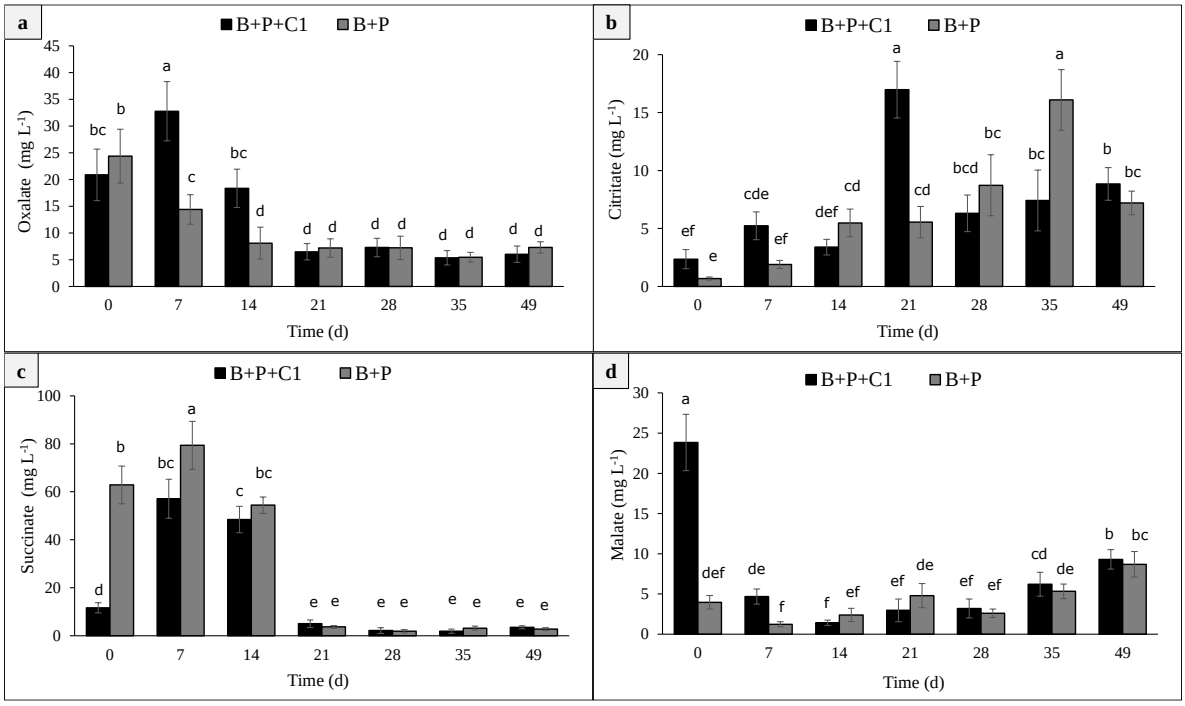


**Figure 5.3.** Root exudations of oxalate (a), citrate (b), succinate (c) and malate (d) by ryegrass plants uprooted from rhizosphere inoculated treatment (B+P+C1) and rhizosphere non-inoculated control (B+P). Each value is the mean of four replicates with error bars representing the standard deviation of the mean.

In addition, in our study we observed that exudation of dicarboxilates such as oxalate and succinate were higher at the beginning of experiment with a later normalization, possibly as

response to the novel stress conditions (**Figure 5.3a and c**). Conversely, levels of complex tricarboxilates such as citrate were gradually increasing after early days of incubation (**Figure 5.3b**). Therefore, it has been suggested that patterns of released OAs change through the time depending on the contamination level and indicate that succinate can have an active role in the removal of organic contaminants such as PAHs (Wang *et al*., 2014), phenomenon that has been currently corroborated by studies about BPS rhizosphere contaminated with pesticides (Diez *et al*., 2015).

### 5.3.4. Quantification of microbial groups via q-PCR

Aiming at evaluating the effect of bacterial inoculation and root influence on some microbial communities existing in the biomixtures contaminated with iprodione, estimations of abundance for total fungi, total bacteria and different bacterial taxa were evaluated via qPCR analyses. Therefore, significant statistical differences between treatment and control through time were observed in these groups, excepting beta-proteobacteria ($p < 0.05$). For total fungi population, it was observed that treatments and controls suffered decreasing levels in their abundances immediately after 7 d of iprodione application (**Figure 5.4a**). As result, it gave us an unclear evidence about the effect of bacterial inoculation and plant root presence, which could be more related to the antifungal action by iprodione than to the above factors. Therefore, these results were not strange to be obtained considering the extensive documentation about the broad antifungal action of iprodione on diverse phytophatogenic fungal species, and the also existing information about negative effect of other fungicides on fungal populations existing in biomixtures (Coppola *et al*., 2011; Marinozzi *et al*., 2013). However, scarce approaches about to detriment action on fungal communities as result of iprodione application have been reported, being decreasing levels of fungal indicators such as ergosterol in soils (Miñambres *et al*., 2009) and affection to mycorrhizal spore germination (Hernandez-Dorrego and Mestre Pares, 2010) the nearest reports available until now. Accordingly, it is possible that antifungal action of iprodione could manifest at higher degrees than those derived from plant or *Arthrobacter* sp. strain C1 presence and consequently masking the real effect of these last two factors.

Likewise, for total bacteria quantification it was observed that abundances were gradually decreasing for every treatment and control through the experiment, with special sensitive effect for unplanted biomixtures. However, normalizing levels after 21 d were observed, overall for B+C1, B+P and B. These results seemed to reflect that the combination of roots and bacterial inoculum delayed the recovery, whereas the single factors were less deleterious for later recoveries (**Figure 5.4b**). Analyzing the different bacterial taxa was observed that actinobacteria (**Figure 5.5**) starting with higher abundances for inoculated treatments strongly possible by amplification of *Arthrobacter* sp. strain C1 that is a Gram-positive actinobacteria. Indeed, was this taxa the most abundant at the beginning of the assay (± 40 – 50 %). However, after time 0 the abundances for treatments and controls were drastically decreased and followed by a gradual normalization, except for B+P+C1 that delayed its evolution (**Figure 5.5**). Thereby, as was explained for total bacteria, actinobacteria were probably more affected under root and bacteria incorporation, being the most affected taxa until 21 d. As opposed, alpha-proteobacteria taxa showed increasing values in every subject, being these more pronounced for B+P+C1 and B+P than for non-rhizosphere biomixtures (**Figure 5.5**). This demonstrated that plant roots improved these values independent from bacterial inoculation that did not enhance the abundances, which placed this taxa as the third highest abundance (± 40 %) from 7 d to 21 d, displacing actinobacteria during this period. About beta-proteobacteria taxa no real changes were visualized through the experiment for any treatment and control which placed this taxa as the second most abundant at 0 time (± 30 %) and the most increasing after that (± 40 %) (**Figure 5.5**). In relation to gamma-proteobacteria abundances, it was showed that their levels were the lowest among the taxa for every treatment and control. Nonetheless, decreasing abundances on early days with later recoveries for everyone did not demonstrate any relation to plants presence or bacterial inoculation (**Figure 5.5**). Finally, for firmicutes increasing values for B+P+C1 and B+P at early days with later recoveries were observed, whereas no changes were noted for B+C1 and B, demonstrating that plant roots improved the abundances better than bacterial inoculation. These values placed this taxa as the fourth most abundant whose highest values were reached during 7d to 21 d (± 5.0 %) (**Figure 5.5**). Thus, summarizing all the above aspect and interactions, we could observe that when taxa decreased their abundances (actinobacteria and gamma-proteobacteria) the combination between plant and bacterial

inoculation delayed the normalization, whereas when increased their levels it was always better related to plant root presence (alpha-proteobacteria and firmicutes), being the inoculation less important.

Comparing the inoculated rhizosphere treatment with non-inoculated rhizosphere control, it was determined that the combination of both factors could affect more the recovery of most taxa than when plants were by themselves. However, rhizosphere samples showed no consistent differences of most microbial groups compared with non-rhizosphere. Thereby, even ryegrass could facilitate the removal of iprodione by releasing root exudates as carbon and energy source, increasing the contaminant bioavailability (via organic acids) or protecting the bacterial inoculum from external injuries (Lefevre *et al*., 2013), taxa abundances differed over time and the differences between treatments became blurred. This fact could be related to the characteristic of the rhizodeposits and the answer by ryegrass under the stress by contamination and overall, the incorporation of the exogenous bacteria into the rhizosphere (Brüggemann *et al*., 2011).

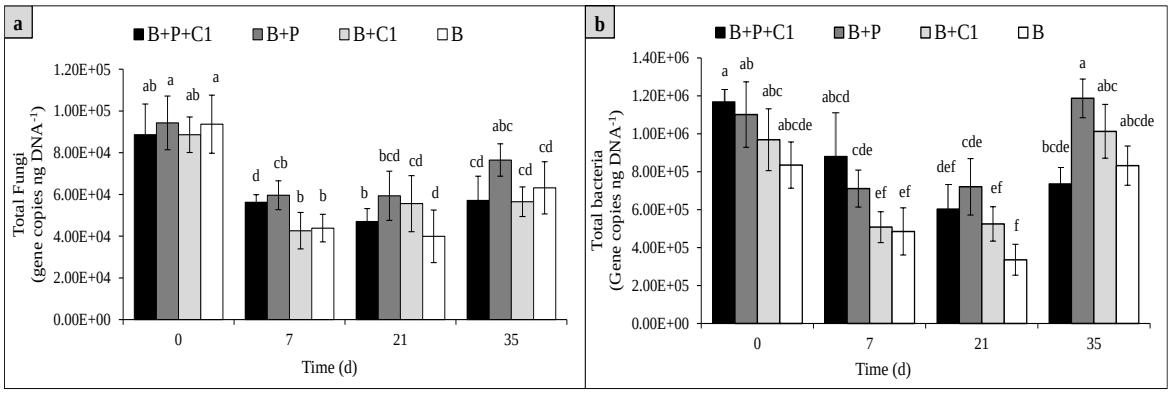


**Figure 5.4.** Estimation of total fungi (**a**) and total bacteria (**b**) abundances determined by qPCR from rhizosphere inoculated treatment (B+P+C1), rhizosphere non-inoculated control (B+P), non-rhizosphere inoculated treatment (B+C1) and non-rhizosphere non-inoculated control (B). Each value is the mean of four replicates with error bars representing the standard deviation of the mean.

Moreover, in our study it was elucidated that inoculation resulted in a better control of iprodione removal process, a main point in bioremediation. However, decreasing abundances

for actinobacteria taxa, where our *Arthrobacter* sp. strain C1 was included, were not correlated with the improvement of removal via bacterial inoculation. Therefore, factors such as adaptation of inoculum in the new environment, the finite nutrient source able to support only a fraction of the biomass (Lenbeau, 2011) and adhesion to clay and organic matter in the biomixture (Secher *et al*., 2013) could hinder the extraction of taxa as time was prolonged nearing values in inoculated treatment with controls. Likewise, Verhagen *et al*., (2013) reported that even when inoculation of BPS with chloropropham- and 3-chroloanilne-degrading bacteria achieved a faster removal of their target contaminants at early steps, the inoculum was not visible after 72 h of inoculation by DGGE analysis, which suggested that the consortium was not dominant during the experiment due to a strong competition with indigenous microorganisms. This phenomenon is a well-documented problem for introduction of exogenous microorganisms, being indicated as the main cause of inoculation failures (Tyagi *et al*., 2011) and the fundamental reason why we incorporated high concentrations of *Arthrobacter* sp. strain C1 as inoculum.

Our results demonstrated that proliferation of some taxa with intrinsic capacity to degrade pesticides could also express the suitable metabolic ability to enhance the removal of iprodione and its metabolites in non-inoculated biomixtures, fact that has been reported for other fungicides (Coppola *et al*., 2011; Tortella *et al*., 2013b) and herbicides (Tortella *et al*., 2013a; Verhagen *et al*., 2015). Nonetheless, the overview seemed to show us that iprodione contamination could be the most preponderant factor exerting negative effects on population of no-target microorganism such as bacterial. Therefore, iprodione application in soil has been demonstrated to disturb moderately populations of actinobacteria and Gram-positive bacteria (Miñambres *et al*., 2009). Similar effect has also been reported for the fungicides tridemorph, vinclozolin (Benerjee and Banerjee, 1991) mancozeb, dinocap (Černohlávková *et al*., 2009) penconazole and cyprodinil (Cocconcelli and Trevisan, 2012) that are able to alter important bacterial taxa involved in soil nitrogen and carbon cycle such as actinobacteria. Accordingly, it is possible that a negative effect of iprodione on actinobacteria population could explain the decreasing value for total bacteria abundances, due to this taxa was the most abundant in our study. Nonetheless, this aspect might be an interesting outlook, because current investigations focused to find new bioindicators (Niepceron *et al*., 2013;

Chang and Chang, 2014) have postulated the sensitive response of actinobacteria taxa as a useful parameter of environmental pollution (Walter *et al*., 2012; Remenár *et al*., 2014).

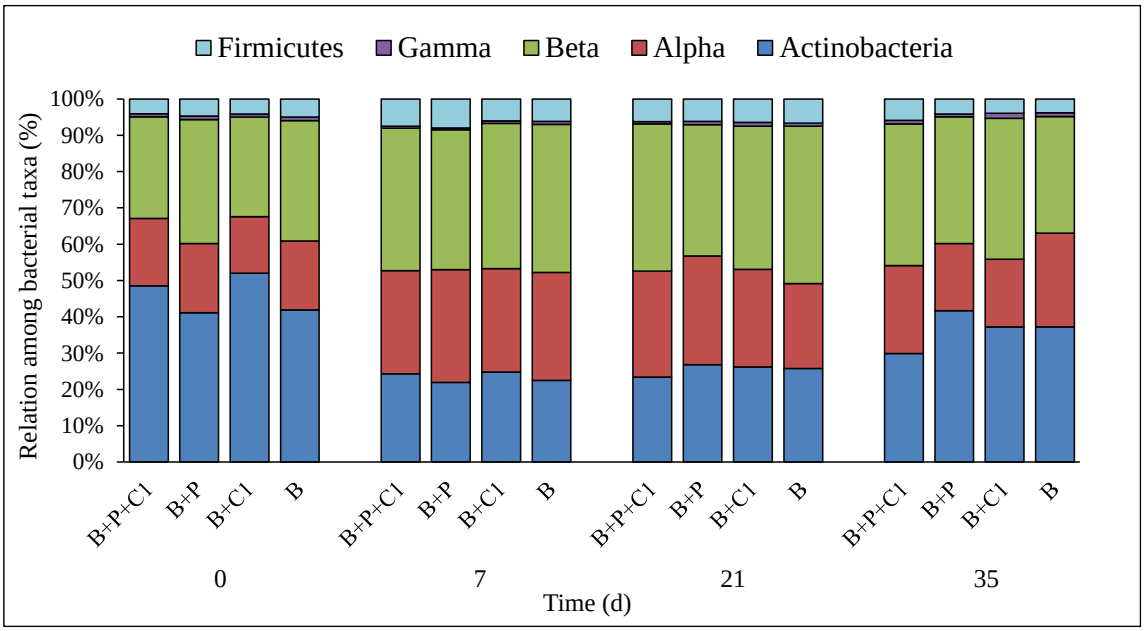


**Figure 5.5.** Relation (%) among abundances of actinobacteria (blue), alpha-proteobacteria (red), beta-proteobacteria (green), gamma-proteobacteria (purple) and firmicutes (light blue) bacterial taxa determined by qPCR. Samples from rhizosphere inoculated treatment (B+P+C1), rhizosphere non-inoculated control (B+P), non-rhizosphere inoculated treatment (B+C1) and non-rhizosphere non-inoculated control (B) were used for analysis at 0, 7, 21 and 35 d of incubation. Each value is the mean of four replicates.

## 5.4. Conclusions

Cover grass rhizosphere is indicated as a key component of BPS where removal of multiple pesticides can be effectively mitigated by a rhizodegrading process. In our study, we demonstrated that inoculation with *Arthrobacter* sp. strain C1 enhanced at least two-fold iprodione DT50s with high production of metabolite I than uninoculated controls, which was in agreement with the characteristics of this strain. On the other hand, negligible detection of iprodione and 3,5-DCA in rhizosphere biomixtures indicated that roots also improved the removal these molecules, overall when rhizosphere was inoculated with strain C1.

Afterwards, the impact of inoculation and roots presence on enzymatic activities did not show a clear pattern during the experiment. Nevertheless, the highest values observed during the most active iprodione removal indicated a highlighted microbial activity at early steps. Similarly, short-chain organic acid exudation noted that plants from inoculated and non-inoculated rhizosphere carried out with higher values of oxalate and succinate at early days coinciding with the most active removal period. After that, the normalization of the levels and increasing values for other organic acids suggested that root exudation played an active role in the removal of iprodione and its metabolites. The analysis of the microbial abundances demonstrated temporal decreasing values for total fungi and total bacteria without any consistence attribution for plant and/or inoculum incorporation, which apparently was more related to stress by iprodione contamination. Nevertheless, bacterial taxa demonstrated better responses when bacterial inoculation and root incorporation were alone than combined, demonstrating that iprodione removal was not determined by microbial abundances. Finally, our results indicated that inoculation with *Arthrobacter* sp. strain C1 and incorporation of ryegrass roots enhanced the removal of iprodione/metabolites and avoided the accumulation of these molecules in biomixture. Nonetheless, bacterial inoculation and the genetic mechanisms involved on iprodione degradation by strains C1 need extra studies in order to develop a suitable inoculum with reliable performance in on-farm biobed systems, aspect that is currently subject of study.

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# Chapter 6

## General Discussion

## General discussion

The present thesis aimed at evaluating the microbial degradation of iprodione and its metabolite 3,5-DCA by a degrading bacterial inoculum and its application in the grass cover of a biobed. In order to reach this objective the study was divided into four different main areas.

In Chapter 2, the isolation and characterization of iprodione- and 3,5-DCA-degrading bacteria from soil with and without historical pesticide applications was investigated. Here, at a first glance to test the iprodione removal by a pristine soil, we observed slow removal rates with scarce production of 3,5-DCA, which was probably a result of the soil acidic pH (5.5) where iprodione has been reported to be more stable (Walker *et al*., 1986; Walker, 1987) than in neutral to alkaline soils (Cayley and Hide, 1980). Hence, in order to increase the chances of isolating iprodione- and 3,5-DCA-degrading bacteria, we incorporated a soil with history of repeated applications of iprodione, supported in the idea that large soil exposition to pesticides is able to induce metabolic adaptations of soil microorganisms as response to the novel stressing conditions (Arbeli and Fuentes, 2007). However, the most suitable degrading subjects were obtained from the pristine acidic soil, indicating that exposition to natural aliphatic and aromatic halogenated compounds as well as the imidazolidinedione moiety present allantoin and uric acid, could confer suitable degrading abilities to these isolates (Gribble, 1992; Copping and Duke, 2007). Therefore, laboratory enrichments left to the isolation of a pure *Pseudomonas* sp. strain able to partially degrade 3,5-DCA (*ca*. 34% degradation) as sole C and N source. On the other hand, was also isolated an iprodione-degrading consortium able to total transform iprodione as sole C and N source, whose posterior purification guided us to the isolation of a pure *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2. In this context, *Pseudomonas* strains have been found to degrade several anilines (Konopka *et al*., 1989), chloroanilines (Vangnai and Petchkroh, 2007) and dichloroanilines (Travkin and Golovleva, 2003), *Arthrobacter* sp. strains have been previously described as iprodione-degraders (Athiel *et al*., 1995) and genus *Achromobacter* have been reported as degraders of carbofuran (Tomasek and Karns, 1989), methyl parathion (Zhang *et al*., 2005) and endosulfan (Li *et al*., 2009). Afterwards, testing

different C and N sources for both pure strains, we demonstrated that *Arthrobacter* sp. strain C1 was the main iprodione degrader from the consortium, whose degrading ability at acidic conditions was in contrast to the *Arthrobacter*-like strain reported by Athiel *et al*. (1995). On the other hand, *Achromobacter* sp. strain C2 was only able to co-metabolize iprodione, being its role in the metabolism of iprodione not clear. In addition, it was noticed that strain C1 was able to release isopropylamine metabolite immediately after iprodione hydrolysis suggesting a possible similitude to the metabolic pathway proposed by Mercadier *et al*. (1997), aspect that was investigated in Chapter 3.

Considering that *Arthrobacter* sp. strain C1 was the main iprodione degrader, different parameters such as pH and T° media as well as the exposition to a mixture of pesticides commonly used in combination with iprodione were investigated. The results demonstrated that iprodione degradation was influenced by changes in pH and T°. Thereby, at high pH and T° it was noted that iprodione was prone to hydrolysis rapidly and independently from bacterial inoculation. This was in agreement with the well documented vulnerability of dicarboxaminde fungicides to hydrolysis at alkaline pH (Szeto *et al*., 1989). However, a large production of 3,5-DCA was only observed for bacterial treatments under these conditions, indicating that bacteria could improve the iprodione degradation via a different pathway from the reported for abiotic hydrolysis (Zadra *et al*. 2006). On the other hand, at acidic pHs bacteria showed fast degrading rates with negligible formation of 3,5-DCA, indicating that low pHs changed the degrading pattern, but did not affect the bacterial performance as it happened with other strains previously reported (Athiel *et al*. 1995). Finally, the co-existence of extra pesticides did not disrupt the bacterial capacity to degrade iprodione, whereas no degradation of any other pesticide was observed, possibly due to the substantial chemical differences among them and the probable specificity of strain C1 to degrade iprodione (Cullington and Walker 1999; Singh and Walker 2006).

In Chapter 3, the efforts were mainly focused on elucidating the metabolic pathway of iprodione by the two purified strains C1 and C2 and identify their role in the transformation steps of iprodione. Here, pure *Arthrobacter* sp. C1 and *Achromobacter* sp. C2 strains were incubated against iprodione and its main reported metabolites; 3,5-dichlorophenyl-carboxiamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II) as the sole or extra C and N sources in MSM and LB, respectively. This study corroborated that

*Arthrobacter* sp. strain C1 was the main iprodione degrader, following a transient formation of isopropylamine and a large accumulation of metabolite I in MSM, whereas no significant degradation was observed for *Achromobacter* sp. strain C2 under the same conditions. Degradation of iprodione in MSM did not appear to stimulate the growth of the two strains which showed a significant decrease in their abundances. Otherwise, full conversion of iprodione to metabolite I, metabolite II and 3,5-DCA was observed for every bacterial strain in LB, indicating that it could be co-metabolized by bacteria. Here, strain C2 showed a relative stable population throughout the incubation compared to strain C1 whose population significantly declined at early steps followed by a gradual increase. These results demonstrated a similar iprodione pathway to the one first described by Athiel *et al*. (1995) and further verified by Mercadier *et al*. (1997). However, the nature of each step involved in the pathway was not described by these researchers, aspect that was focus of our study.

Posterior experiments carried out with metabolite I and metabolite II demonstrated that bacteria were unable to catabolize metabolite I as the sole C and N source, whereas no difficulties to co-metabolize both molecules were observed. This capacity of both strains to only co-metabolize metabolite I contrasted with findings by Mercadier *et al*. (1997) who suggested that this step is mostly abiotic and it can only be accelerated by bacteria. On the other hand, a particular reduction in the capacity of *Arthrobacter* strain C1 to co-metabolize this molecule compared with its catabolic rate was observed, fact that could be attributed to the oligotrophic character of *Arthrobacter* genus (Poindexter 1981). In addition, *Arthrobacter* sp. strain C1 showed decreasing abundances with later recoveries, whereas no real changes in *Achromobacter* sp. strain C2 population were observed during the exposition to metabolite I and II, which provided the first evidence for a toxic effect of metabolite I to our main iprodione degrading strain C1. Here, recent proteogenomic analysis of pesticide-degrading bacteria exposed to high pesticide concentrations have demonstrated to trigger an intracellular toxicity response to the cells (Breugelmans *et al*. 2010; Bers *et al*. 2011) suggesting that pesticide degradation could be a detoxification mechanism, especially during co-metabolic processes.

Furthermore, in Chapter 3 we discovered that *Arthrobacter* sp. strain C1 was able to degrade vinclozolin and propanil, pesticides that have a similar chemical structure to iprodione, which constitutes the first report describing a cross degradation among these

pesticides. Thereby, our strain showed ability to enhance the transformation of vinclozolin into 3,5-DCA and propanil into 3,4-DCA as final metabolites, being coincident with previous reports about vinclozolin (Golovleva *et al*., 1991; Cain and Mitchell, 1996; Lee *et al*., 2008) and propanil (Hirase and Matsunaka, 1991; Zhang *et al*., 2011; Herrera-González *et al*., 2013) degrading bacteria. Therefore, this cross degradation could be possibly via cleavage of the carboxamide bond common between iprodione and vinclozolin (Pospíšil *et al*., 1999) and by expression of a hydrolase enzyme able to degrade the N´-substituent propionate present in propanil (Cullington *et al*. 1999).

Afterwards, in order to obtain more information about the potential use of *Arthrobacter* sp. C1 and *Pseudomonas* sp. strains to formulate a degrading inoculum, in Chapter 4 we aimed at giving an approach to the potential affecting factors for the rhizodegradation of iprodione and 3,5-DCA by the degrading strains. Here, parameters such as cell growth rates, utilization of different C and N sources, antagonistic effect of soil indigenous microorganisms on our bacterial strains and tolerance of ryegrass (*Lolium perenne*) seeds against higher concentrations of iprodione and 3,5-DCA, were aspects to be considered. In this chapter it was demonstrated that incorporation of simple C source such as sodium citrate could improve the cell growth of *Arthrobacter* sp. C1 and *Pseudomonas* sp. strain, accompanied by faster degradation of their target contaminants, whereas the incubation in presence of a complex pool of nutrients from a soil extract medium delayed these parameters. The enhanced degradation was well relate to the increasing bacterial biomass resulting from the utilization of simple C sources (e.g. sodium citrate), whereas delays were strongly suggested as result of a difficulty for bacterial growth via use of complex nutrient sources present in soil extract medium (Strong *et al*., 2002; Vangnai and Petchkroh, 2007) and the possible presence of catabolic repressors such as antibiotics and xylose in this (Biesterveld *et al*., 1994). However, in the case of 3,5-DCA degradation by *Pseudomonas* sp., it did not improve the results showed previously in Chapter 2, being this the main reason to test different C and/or N sources in order to enhance its degrading rate. Thereby, even when *Pseudomonas* sp. strain was incubated in a rich medium such as LB broth, degradation of 3,5-DCA was not dramatically higher than the previous experiments, possibly as result of its limited metabolic ability to degrade this resistant molecule (PBT Profiler). As results, *Pseudomonas* sp. strain was discarded for future rhizodegrading experiments due to the

unsatisfactory attempts to increase its 3,5-DCA degradation at laboratory scale. The antagonistic effect of soil microorganisms on the growth of our degrading strains demonstrated to be not impediment for their development. However, this only served as a referential view due to the use of cultivable soil microorganisms under controlled laboratory conditions and not under natural environments (Duquenne *et al*., 1999), which in no case, could be interpreted as a reliable approach to the real antagonism or competition against indigenous microorganisms present in biomixture (Thompson *et al*., 2005). The tolerance of ryegrass seeds against higher concentrations of iprodione and 3,5-DCA was carried out with excellent germination indexes, indicating that chemical nature of both contaminants were not deleterious for the development of plants, even at concentration 100 times over the normal application on fields. These results were in agreement with evidences reporting that pre-treatment of crop seeds with iprodione have no negative effect on seed germination, conversely improving germination by prevention of infection with phytophatogenic fungi (Thomas and Sweetingham, 2003; Bankole and Joda, 2004; Rao *et al*., 2009). Thus, due to the suitable performances observed for *Arthrobacter* sp. strain C1 and ryegrass seeds on the above experiments, these subjects were chosen as the main components for a posterior rhizodegrading experiment.

In Chapter 5 the removal of iprodione by the *Arthrobacter* sp. degrading strain C1 inoculated into the grass cover of a biobed was the central aspect to be evaluated. Therefore, to have an integral view about the rhizodegrading process of iprodione by bacterial inoculation, parameters such as removal rates, biological activities, root exudation of short-chain organic acids (OAs) and microbial quantification by qPCR were useful indicators for us. It is well known that the role of plants and rhizosphere degrading microorganisms are the key to reach a successful removal in rhizodegradation (Gerhardt *et al*., 2009). However, the incorporation of pesticide-degrading bacteria into biobeds has been a strategy poorly explored, being the inoculation with chloropropham and 3-chloroaniline-degrading bacteria by Verhagen *et al*., (2013) our nearest and promising referent. Thereby, in our study we demonstrated that removal rates, expressed as $DT_{50}$, were always better when inoculation of *Arthrobacter* sp. strain C1 was incorporated into biomixture, overall when bacterial inoculation and ryegrass rhizosphere were combined. In this context, previous studies in soil (Buyanovsky *et al*. 1995; Korade *et al*. 2009) and biomixture (Diez *et al*. 2015) have

documented the positive effect of rhizosphere on the degradation of pesticides, aspect that support our findings. On the other hand, iprodione removal was also well performed in biomixtures without plants or bacterial inoculum, indicating that indigenous microorganisms were able to carry out suitable removal rates, a central aspect for the formulation of biopurification systems (Sniegowski *et al*., 2011). Furthermore, visualization of large amounts of metabolite I in inoculated biomixtures demonstrated to be coincident with the characterization of our *Arthrobacter* sp. strain C1 at laboratory scale (Campos *et al*., 2015; Chapter 3), whereas scarce visualization of 3,5-DCA in planted samples reflected an active role of plant roots to remove this metabolite possibly via some non-elucidated rhizospheric process such as absorption or immobilization (Gerhardt *et al*., 2009). Regarding the biological activities, no clear patterns attributed to the bacterial inoculation and/or presence of ryegrass roots were possible to be established (Perucci *et al*., 2000; Lin *et al*., 2011) instead being it more related to the iprodione contamination because high levels on this parameter were coincident with the highest period of iprodione removal at early days. Likewise, high root exudation of OAs were not clearly attributed to presence or absence of bacterial inoculum in rhizosphere. Nonetheless, the stress caused by contamination with iprodione could be the best explanation for the higher OAs exudation (Ryan *et al*., 2001; Chaudhry *et al*., 2005), because these root exudates have been documented as suitable molecules to improve the bioavailability of retained contaminants in matrices with high organic matter content, such as our biomixture (White *et al*., 2003; Ling *et al*., 2009).

Finally, the analysis of microbial abundances showed that bacteria as well as fungi populations were decreased temporarily during the experiment without attribution again to the bacterial inoculation or plant root presence, suggesting that iprodione exposition was the main affecting factor. This effect was not strange due to the antifungal property of iprodione (Hernandez-Dorrego and Mestre Pares, 2010) with transient negative effect on soil fungi (Miñambres *et al*., 2009), whereas affection to bacteria could be related by action to non-target microorganisms such as it has been reported for other fungicides (Benerjee and Banerjee, 1991; Černohlávková *et al*., 2009; Cocconcelli and Trevisan, 2012). Nevertheless, the analyses of different bacterial taxa demonstrated that combination of bacterial inoculation and rhizosphere could affect more the recovery of most taxa than when plants were by themselves, which could be related to the answer of ryegrass under the stress by

contamination and overall, the incorporation of the exogenous bacteria into the rhizosphere (Brüggemann *et al*., 2011). In addition, decreasing abundances for actinobacteria taxa (where our *Arthrobacter* sp. strain C1 is included) suggested that the inoculum was not dominant during the experiment due to a strong competition with indigenous microorganisms (Tyagi *et al*., 2011; Verhagen *et al*., 2013), which however did not affect the removal rates of iprodione and metabolites indicating that this parameter was not affected by the microbial abundance.

Thus, considering all the above mentioned statements, incorporation of *Arthrobacter* sp. strain C1 as inoculum into rhizosphere of a biobed could result in an improvement for iprodione/metabolite removal rates. However, deeper studies aiming at the formulation of a stable and reliable inoculum able to support the stressing conditions ruling in rhizosphere, are necessary to turn it into a reliable alternative to use in on-farm biobed systems. Additionally, further genomic/transcriptomic analysis of strain C1 are currently being carried out in order to identify the genetic elements involved in the different steps of the metabolic pathway of iprodione, which could mean in a better understanding about nature of our bacterial inoculum and its use as a biorremedial tool.

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# Chapter 7

## General Conclusions

## General conclusions

Iprodione is a very popular fungicide used in a range of crops whose microbial degradation constitute the key process for its control in the environment. In the present work we reported the isolation of an iprodione-degrading culture and of a 3,5-DCA-degrading bacterial strain from an acidic pristine soil without previous exposure to the fungicide. The latter showed only partial degradation of 3,5-DCA and was identified as *Pseudomonas* sp., whereas iprodione-degrading culture was composed of two bacterial members identified as *Arthrobacter* sp. strain C1 and *Achromobacter* sp. strain C2. Posterior studies revealed that strain C1 was the key iprodione-degrading strain whose role in formation of intermediate metabolites such as isopropylamine from the initial hydrolysis of iprodione indicated its probable metabolic pathway. Characterization of strain C1 showed that it was able to maintain its degrading capacity in a wide range of temperature and pH and in the co-presence of a mixture of other pesticides expected to be found along with iprodione in on-farm biobed systems.

The elucidation of the iprodione metabolic pathway by the two purified strains C1 and C2 and the identification of their roles in the transformation steps of iprodione demonstrated that bacteria exhibited variable nutritional requirements but employed the same metabolic pathway. Strain C1 was the unique strain able to utilize iprodione and metabolite II as C and N sources, whereas it could only co-metabolize metabolite I, being this latter step apparently a detoxification process. On the other hand, strain C2 was only able to co-metabolize iprodione and its intermediate metabolites. Therefore, our data suggest that the bacterial transformation of metabolite I appears to be the rate-limiting step in the metabolic pathway of iprodione. Additionally, strain C1 demonstrated capacity to metabolize the structurally related vinclozolin and propanil, but not procymidone and phenylureas pesticides, suggesting a specificity of the catabolic enzymes involved in the degradation of iprodione.

The approach to the potential affecting factors for rhizodegradation of iprodione and 3,5-DCA by degrading strains revealed that these were better performed in presence of simple C carbon sources than complex nutrient sources from a soil extract medium. However, *Pseudomonas* sp. strain did not substantially improve its 3,5-DCA degrading rate in presence

of different C and N sources, possibly a limited metabolic ability to degrade this molecule, discarding the utilization of this strain as a bacterial inoculum for posterior biorremedial studies. Afterwards, the antagonistic effect of indigenous soil microorganisms seemed not affect the growth of *Arthrobacter* sp. strain C1 and *Pseudomonas* sp. However, results reflected a restrictive spectra of the possible adverse effect by competition with the natural soil microflora being interpreted only as a reference. In addition, testing the tolerance two different varieties of *Lolium perenne* seeds to germinate in presence of high levels of iprodione and 3,5-DCA, it was demonstrated that varieties had relatively high tolerance to the contaminants, considering them as suitable subjects to be incorporated together *Arthrobacter* sp. strain C1 in future rhizodegrading experiments.

The enhancing of iprodione removal by *Arthrobacter* sp. degrading strain C1 inoculated in the grass cover of a biobed, noticed that removal rates of iprodione and metabolites were improved notoriously by inoculation with strain C1. Nevertheless, the rates where enhanced even more when bacterial inoculation was done in presence of rhizosphere biomixture, indicating that root were important to remove these molecules. In this context, increasing biological activities and root exudation of organic acids at the beginning of experiment, revealed that microorganisms and roots had active roles in the removal of iprodione and intermediates. However, erratic patterns for these parameters could not clarify the responsibilities of bacterial inoculation and/or plant presence in the process. Finally, the analysis of the microbial abundances demonstrated temporal decreasing populations for total fungi and total bacteria without any consistence attribution for plant and/or inoculum incorporation, which apparently was more related to stress by iprodione contamination. Nevertheless, bacterial taxa demonstrated better responses when bacterial inoculation and root incorporation were alone than combined, demonstrating that iprodione removal was not determined by microbial abundances.

Thus, our study demonstrated that *Arthrobacter* sp. strain C1 is an interesting iprodione-degrading subject, whose potential incorporation into the cover grass of biopurification systems such as biobed might be a suitable strategy to enhance the removal of iprodione and its intermediate metabolites. Nevertheless, studies oriented to transform *Arthrobacter* sp. strain C1 in a real biorremedial alternative seems to be necessary investigated in deep. Therefore, formulation of a stable and versatile inoculum to be used under field conditions

and the elucidation of genetic and enzymatic mechanisms involved in the iprodione metabolism by strain C1 are being focus of our current investigation.