Doctoral Thesis

Green fluorescent protein (GFP) 
a tool to study root interactions in mixed plant stands

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GREEN FLUORESCENT PROTEIN (GFP)
A TOOL TO STUDY ROOT INTERACTIONS IN MIXED PLANT STANDS

A dissertation submitted to

ETH ZURICH

For the degree of

Doctor of Sciences

Presented by

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2009
[...] toute l'invention consiste à faire quelque chose de rien.
Racine (1670)
# Table of content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENT</td>
<td>1</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>III</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>1</td>
</tr>
<tr>
<td>RESUME</td>
<td>3</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>5</td>
</tr>
<tr>
<td><strong>Maize as a plant model for root research</strong></td>
<td>5</td>
</tr>
<tr>
<td>Maize in the world</td>
<td>5</td>
</tr>
<tr>
<td>Maize cropped in living mulches</td>
<td>6</td>
</tr>
<tr>
<td><strong>Root systems</strong></td>
<td>7</td>
</tr>
<tr>
<td>The root system of Maize</td>
<td>7</td>
</tr>
<tr>
<td>The root system of Italian ryegrass</td>
<td>8</td>
</tr>
<tr>
<td><strong>Root interactions</strong></td>
<td>9</td>
</tr>
<tr>
<td>Methods to study roots</td>
<td>10</td>
</tr>
<tr>
<td>General overview</td>
<td>10</td>
</tr>
<tr>
<td>Methods to study root interactions</td>
<td>11</td>
</tr>
<tr>
<td>Hypotheses and objectives</td>
<td>13</td>
</tr>
<tr>
<td><strong>Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.</strong></td>
<td>15</td>
</tr>
<tr>
<td>Abstract</td>
<td>15</td>
</tr>
<tr>
<td>Introduction</td>
<td>16</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>17</td>
</tr>
<tr>
<td>Components of a minirhizotron imaging system based on a webcam</td>
<td>17</td>
</tr>
<tr>
<td>Comparison of webcams</td>
<td>21</td>
</tr>
<tr>
<td>Plant material</td>
<td>21</td>
</tr>
<tr>
<td>Results and discussion</td>
<td>22</td>
</tr>
<tr>
<td>Development of a minirhizotron imaging system</td>
<td>22</td>
</tr>
<tr>
<td>Adaptation of the imaging system to identify fluorescent roots</td>
<td>24</td>
</tr>
<tr>
<td>Conclusion</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER 2: THE USE OF GREEN FLUORESCENT PROTEIN (GFP) AS A TOOL TO IDENTIFY ROOTS IN MIXED PLANT STANDS 27

ABSTRACT 27
INTRODUCTION 28
MATERIAL AND METHODS 30
PLANT MATERIAL 30
EXPERIMENTAL CONDITIONS 31
IMAGING EQUIPMENT 32
SCREENING OF ROOTS GROWING IN SOIL 34
RESULTS 35
EXPRESSION OF GFP ALONG ETH-M72GFP ROOTS IN POUCHES 35
ROOT SCREENING OF ETH-M72GFP GROWN ALONE IN THE SOIL 35
DISCRIMINATION OF ROOTS BETWEEN INTERSPECIFIC NEIGHBOURS 39
DISCUSSION 42

CHAPTER 3: ROOT GROWTH OF MAIZE IN A LIVING ITALIAN RYEGRASS MULCH 47

ABSTRACT 47
INTRODUCTION 48
MATERIAL AND METHODS 50
PLANT MATERIAL 50
EXPERIMENTAL CONDITIONS 50
SAMPLING AND SCREENING OF DATA 52
DATA ANALYSIS 53
RESULTS 54
EFFECTS OF ITALIAN RYEGRASS ON THE SHOOT AND ROOT GROWTH AT THE ANTHESIS OF MAIZE 54
EFFECTS OF ITALIAN RYEGRASS ON THE SPATIAL DISTRIBUTION OF MAIZE ROOTS AT ANTHESIS 55
DYNAMICS OF THE ROOT GROWTH OF MAIZE IN BARE SOIL AND IN THE LIVING MULCH 58
DISCUSSION 60
CONCLUSIONS 62

GENERAL CONCLUSION AND OUTLOOK 63

GENERAL CONCLUSION 63
OUTLOOK 65

REFERENCES 68

ACKNOWLEDGEMENTS 75

CURRICULUM VITAE 76
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOV</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BS</td>
<td>Bare Soil</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Coupled Devices</td>
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<tr>
<td>CMOS</td>
<td>Complementary Metal Oxide Semi-conductor</td>
</tr>
<tr>
<td>d.f.</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>ITS</td>
<td>Image Time Serie</td>
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<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
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<tr>
<td>K₂O</td>
<td>Potassium Oxide</td>
</tr>
<tr>
<td>LED</td>
<td>Light Emitting Diode</td>
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<tr>
<td>LM</td>
<td>Living Mulch</td>
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<tr>
<td>lm</td>
<td>Linear Model</td>
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<tr>
<td>lx</td>
<td>Lux</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
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<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NH₄</td>
<td>Ammonium</td>
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<tr>
<td>NH₄NO₃</td>
<td>Ammonium nitrate</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>P₂O₅</td>
<td>Phosphorus pentoxide</td>
</tr>
<tr>
<td>p</td>
<td>Probability level</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl Chloride</td>
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<tr>
<td>S</td>
<td>Sulfur</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>USB</td>
<td>Universal Serial Bus</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
Summary

Maize (*Zea mays* L.) is, with more than 158 millions hectares, the most cultivated crop in the world. For conventional cropping the soil is tilled rather intensively and seeds are sown in rows into the bare soil. Though this system is most efficient in terms of grain yield, it has a negative environmental impact associated to soil erosion and pollution of water sources. The cultivation of maize in living mulch systems has been proposed as an environmentally sound option for suppressing weeds and minimizing soil erosion. Despite the environmental advantage of living mulch systems, maize grain yield is significantly reduced under such cropping system. Since the shoot competition between maize and the cover crop is usually controlled, the poor performance of maize was attributed to below-ground interactions that have not been assessed in detail.

Roots are fundamental for the anchorage, water and nutrient uptake of plants. Root systems usually share the soil with intra- or interspecific neighbors. Due to methodological constraints, only few studies measured root parameters of coexisting plants. This knowledge is crucial to understand belowground interactions of plants in the soil, to determine the precise location of roots of different plants in space and time and thus to understand and manage the coexistence of plants in natural and managed ecosystems.

The overall objectives of this thesis were: i) to develop a method which allows to identify and localize roots from different plants into the soil, and ii) to apply this method to understand root interactions between maize and Italian ryegrass (*Lolium multiflorum* Lam.) in a living mulch system.

In a first step it was attempted to develop a method that allows identifying roots from different plants in the soil. For this purpose, ETH-M72\(_{GFP}\) maize plants expressing the green fluorescent protein (GFP) in its roots were chosen. To detect these roots in images obtained from minirhizotron (i.e. transparent tubes inserted in the soil) it was necessary to develop and construct a new minirhizotron camera system. This camera was constructed using affordable and easily obtainable materials and allowed for detecting fluorescent roots from transgenic maize expressing
Summary

GFP (ETH-M72\textsubscript{GFP}). This was the first minirhizotron imaging system that allows detecting fluorescent roots and thus identifying roots in mixed plant stands.

Quantification of fluorescent roots in minirhizotron images was a powerful approach to study the relative distribution of roots in mixed plant stands. This approach was validated in experiments using ETH-M72\textsubscript{GFP} with its corresponding wild type, Italian ryegrass, and soybean (Glycine max (L.) Merr.). We demonstrated that a transformed plant expressing a fluorescent protein combined with the minirhizotron technique allows distinguishing roots from different intra-specific or inter-specific plants in the soil. It was possible to recognize roots, even fine ones, and to precisely determine their relative position. This method allows studying the dynamic interaction of roots from different plants throughout space and time and it provides the urgently needed method for the simple, inexpensive, non-destructive, and objective assignment of roots in mixture of plants.

Understanding below-ground interactions is fundamental to identify opportunities to increase the grain yield of maize in living mulch systems. An experiment was conducted growing ETH-M72\textsubscript{GFP} alone or together with Italian ryegrass in a living mulch system. The root growth and relative distribution were followed using the newly developed method, and the shoot biomass was harvested at anthesis. Maize biomass was strongly decreased by the presence of Italian ryegrass. The maize root density and leaf area were 41\% and 39\% lower in the living mulch maize than in the mono-cropped maize. Italian ryegrass decreased strongly the root density of maize, but without affecting the patterns of its spatial distribution. The proliferation of the root system of maize was delayed by the presence of Italian ryegrass and this effect was especially evident in the area where the shoot of Italian ryegrass was not suppressed.

In conclusion, the outlined method allows unravelling fundamental belowground ecological processes and it was used here to obtain unique and novel results about below-ground interactions of two different species. Because transgenic constructs expressing fluorescent proteins in different colors are available, a new avenue for studying complex belowground plant interactions in natural and managed ecosystems is now open.
Résumé

Couvrant plus de 158 millions d’hectares, le maïs (Zea mays L.) est la plante la plus cultivée du monde. Traditionnellement le maïs est semé sur un sol nu après labour. Bien que ce système permette de bonnes récoltes, il engendre de nombreuses contraintes environnementales associées à l’érosion des sols et à la pollution des eaux. Le semis de maïs dans une culture de couverture a été proposé comme alternative pour limiter l’érosion, la pollution des sols et le développement des mauvaises herbes. Malgré l’avantage environnemental de ces semis sous couverture, ils conduisent souvent à une diminution des rendements en grain. La compétition pour la lumière étant normalement limitée grâce à un contrôle des parties aériennes de la culture de couverture, les faibles rendements doivent être attribuées aux interactions ayant lieu dans le sol et qui n’ont pas encore pu être étudiées en détail.


Les principaux objectifs de cette étude étaient : i) de développer une méthode permettant d’identifier et de localiser les racines de différentes plantes dans le sol, et ii) d’appliquer cette méthode pour comprendre les interactions racinaires entre le maïs et le ray-grass italien (Lolium multiflorum Lam.) installé comme plante de couverture.

L’objectif de la première partie a été de développer une méthode permettant d’identifier les racines de différentes plantes dans le sol. Pour cette étape, des plants de maïs ETH-M72_{GFP} exprimant la protéine fluorescente verte (GFP) au niveau racinaire ont été sélectionnés. Afin de détecter ces racines dans des images issues de minirhizotron (i.e. tubes transparents insérés dans le sol), nous avons développé et construit une nouvelle caméra. Cette caméra a été construite en
utilisant des composants abordables et facilement trouvables pour détecter les racines fluorescentes du maïs transgénique exprimant la GFP. Il s’agit du premier système d’imagerie permettant de détecter des racines fluorescentes et ainsi d’identifier les racines dans un peuplement mixte. Cette approche a été validée au travers d’expérimentations utilisant le maïs ETH-M72<sub>GFP</sub> et son correspondant « naturel », ray-grass italien et le soja (<i>Glycine max</i> L. Merr.). Nous avons ainsi démontré qu’une plante transformée exprimant une protéine fluorescente combinée avec la technique du minirhizotron permettait de distinguer les racines de différentes plantes intra- ou interspécifiques dans le sol. Il a été possible de reconnaître les racines, même les fines, et de spécifier leurs positions relatives. Cette méthode permet d’étudier la dynamique des interactions racinaires de différentes plantes dans l’espace et le temps, et cela répond au besoin urgent de bénéficier d’une méthode simple, abordable et non destructive pour l’étude des racines d’un peuplement mixte.

La compréhension des interactions ayant lieu dans le sol est fondamentale afin d’apporter des solutions pour augmenter les rendements en maïs grain sous culture de couverture. Nous avons conduit une expérimentation dans laquelle le maïs ETH-M72<sub>GFP</sub> était associé à une culture de ray-grass italien. La croissance racinaire et la distribution relative des racines ont été suivies en utilisant cette nouvelle méthode, et la biomasse aérienne a été récoltée à la floraison. La biomasse du maïs a été fortement diminuée par la présence de ray-grass italien. La densité racinaire du maïs et sa surface foliaire étaient respectivement 41% et 39% plus faibles en association avec le ray-grass italien en comparaison avec la monoculture. Le ray-grass italien diminue fortement la densité racinaire du maïs sans affecter sa distribution spatiale. La croissance du système racinaire du maïs a été retardée par la présence du ray-grass italien et cet effet était plus évident dans les zones où la partie aérienne de la plante de couverture n’avait pas été supprimée.

En conclusion, cette nouvelle méthode permet d’appréhender des processus écologiques fondamentaux ayant lieu dans le sol et elle a été utilisée pour obtenir des résultats originaux sur les interactions racinaires de deux espèces. La disponibilité de constructions transgéniques exprimant des protéines fluorescentes de différentes couleurs permet d’envisager l’étude d’interactions racinaires plus complexes au niveau d’une communauté dans des écosystèmes naturels ou cultivés.
Maize as a plant model for root research

Maize in the world

Maize (Zea mays L.) is the most produced crop in the world; its world production is 791 millions of tons in 2007, (FAOSTAT 2007). It was domesticated between 7 000 - 10 000 years ago in North America, more probably southwestern or south central Mexico (Goodman, 1988) from Mexican teosinte (Zea mays subsp. parviglumis). The propagation of maize culture started first on the southern and northern American continent and has been spread since the XVI century to Europe, Africa, Asia and Oceania. In Europe, maize was introduced by Columbus after his arrival to Spain in 1493, the first illustrations of maize in Europe were published in 1534 in Venice (Sauer, 1960). Maize is an annual cereal with a large morphological diversity among its varieties. There are more than 300 maize races (Taba, 2003) with maturation times that range from 60-70 days to 11 months and plant heights from 0.30 m to 10 m. Maize can grow at different altitudes and climates, and as a consequence of breeding during the 20 century, it can be cultivated to rather high latitudes such as 55° in both southern and northern hemisphere (Shaw, 1988).

Utilizations of maize vary depending on the economical development of the countries. In developing countries maize is more often used for direct human consumption, whereas in industrialized countries the main use of maize is to feed cattle and to produce processed products. As a consequence, maize is an essential crop for the global food security and an important industrial input.

Because of its high economic importance, more than 158 millions hectares are used worldwide to crop maize. For the conventional cropping of maize the soil is tilled rather intensively and maize is sowed in rows into the bare soil. The conventional cropping is the most efficient in terms of grain yield, but it has negative environmental impacts.
Maize cropped in living mulches

Leaving the soil bare in the conventional cropping of maize is associated to soil erosion and pollution of water sources by the fertilizers and the agrochemicals (Ploey 1989). In addition, the soil water content is reduced by higher water evaporation. These negative effects can be reduced by having the soil covered. Different alternatives exist to cover the soil, such as intercropping systems. Andrews and Kassman (1976) define an intercropping system where a crop is grown simultaneously with one or more species. Another alternative is to use cover crops as living mulches: “A cover crop is any living ground cover that is planted into or after a main crop and then commonly killed before the next crop is planted. Living mulches are cover crops planted either before or with a main crop and maintained as a living ground cover throughout the growing season (Hartwig and Ammon, 2002).“

Cover crops reduce soil erosion and thus have a direct positive effect on the soil productivity. When maize is planted into living mulches, agrochemicals loss and water runoff can be reduced by 95% compared with the conventional practice (Hall et al., 1984). The cover crops add organic matter into the soil which increases the soil productivity (Bullock, 1992). Soil tilth is also improved due to the formation of biopores (Nakamoto, 2000) where roots can grow (Rasse and Smucker, 1998) and this improve soil structure (Reicosky and Forcella, 1998) and increase soil productivity. The pollution of water sources resulting from nitrogen loss is reduced with cover crops (Danso et al., 1991; Randall et al., 1997; Rasse et al., 2000). Maize also benefits from the nitrogen release by the cover crop, especially if it is a legume (Ebelhar et al., 1984; Fox and Piekielek, 1988; Hargrove, 1986). Living mulches also have a role in weed control, which in terms of efficiency can be comparable to a commercial herbicide (Degregorio and Ashley, 1985).

Despite the environmental advantages of using living mulches, there is a significant decrease in the grain yield of maize (Garibay et al., 1997; Liedgens et al., 2004b; Martin et al., 1999). When two plants grow in the same space, there is an interaction between them. Competition appears at different levels and can empirically be split in an above and belowground competition. Plant competition takes place when the resources are shared (Grace and Tilman, 1990), and different levels and indices of plant competition can be measured (Weigelt and
General Introduction

Jolliffe, 2003). The aboveground competition is essentially for light, and can be easily studied. However, the shoot competition in living mulches is avoided by mechanical or chemical control. In contrast, the belowground competition is difficult to study due to its little accessible environment. Furthermore, roots have to take up with the exception of light and carbon, all the resources needed for the growth, survival and fitness of plants.

**Root Systems**

Roots are primordial in plant development but due to the fact of their relatively inaccessibility they were much less studied than shoot. Besides their major function of anchorage, absorption, and transport of water and nutrients, roots have, depending on the species, secondary functions like storage, aerial absorption, and aeration for the pneumatophores (Bell and Bryan, 1991).

“Root systems are composed of the primary root that originates as part of the developing embryo in the seed, postembryonic, shoot-borne roots, and lateral roots that emerge from all root types” (Jackson and Daniel, 2005). This study will focus principally on the maize root system as a plant model for growth in living mulches of Italian ryegrass.

*The root system of Maize*

Maize (*Zea Mays*) forms a complex root system (Fig1) comprising embryonic and post-embryonic roots. The embryonically formed root system is made of the primary root and a variable number of seminal roots (Hochholdinger et al., 2004). The primary roots appear on successive phytomers until the flowering period (Girardin et al., 1986; Hoppe et al., 1986). However as the development progresses, the post embryonic shoot-borne root system becomes dominant and responsible together with its lateral roots (i.e. root branches) for the major portion of water and nutrient uptake. The morphology of the root system of a maize plant in an advanced developmental stage can be described by the nomenclature of Girardin et al (1986) and the different types of roots are referred as: primary roots, adventitious roots and lateral roots (Kozinkka, 1992). Although, there are many different ways to refer to root types, one that may integrate them all is the one that refers to roots as “axile roots” and “lateral roots” (Cahn et al.,
General Introduction

1989). Fine roots become especially important for the uptake of nutrients and water (Zobel, 2003). The main function of the root system is to explore the soil, adopting very different spatial distributions. The growth of maize root decreases strongly with the depth and the distance from the plant (Barber, 1971a).

The root system of Italian ryegrass

Italian ryegrass has an expansive and dense fibrous root system (Fig1) (Liedgens et al., 2004a). It can tolerate temporary floods but has a better growth when the soil is well drained (Miller, 1984). It responds positively to N and P supply. When Italian ryegrass is used as a cover crop of a living mulch system, it covers efficiently the soil surface, minimizing its erosion and reduces significantly the N loss by leaching (Liedgens et al., 2004b).

Figure 1: Drawings of excavated root systems (Kutschera, 1960).
A: Maize; B: Ryegrass.
Root interactions

When two or more plants species grow together, they share the same soil volume and therefore the same resources. Ricklefs and Miller (1999) defined root competition as reductions in the availability of soil resources for a root caused by another root. Competition belowground is more complex than aboveground where competition is mainly for light. Belowground, the competition is essentially for water, nutrients and space (Casper and Jackson, 1997). Plants have to arrange and rearrange the roots depending on the conditions to increase their access to the soil resources (Robinson, 1994). Therefore plant growth depends on the ability of a plant to capture nutrient and water and this is directly associated to the spatial root distribution.

The response of root systems to the soil environment is conditioned by the presence of neighbors. Plant species respond in different ways depending if they are grown in intraspecific or interspecific competition; plants have the capacity to recognize between self and non-self neighbors and inhibit the root growth in the presence of roots from the same individual (Falik et al., 2006; Murphy and Dudley, 2009). Architectural adjustments can improve the capture of resources. Plants increase the efficiency in which the soil is explored by means of root segregation. This was observed by supplying nutrients (Chassot et al., 2001) and water (Machado and Oliveira, 2003) in a localized fashion. This segregation of the root system can be temporal or a vertical or horizontal spatial segregation (Hutchings et al., 2003). Architecture of root systems can anyway vary depending on species and present a high plasticity (Osmont et al., 2007).

Higher root proliferation increases the capture of soil resources (Fransen et al., 1999). The relative distribution of roots also affects the capture of soil resources; roots which are at the surface will be more efficient because a higher access for the nutrient and the water (van Wijk and Bouten, 1999).

In living mulches, one of the strongest competitions is for the water, maize biomass in living mulches is most reduced in a dry year (Carreker et al., 1972; Kurtz et al., 1952). Therefore living mulches set a high level of competition for water with maize, except under wet conditions (Echtenkamp and Moomaw, 1989). A further major competitive factor is N (Feil et al., 1997). The lesser the available resources, the higher the competition is (Pugnaire and Luque, 2001). As a
consequence, maize cropping is only possible in living mulches by reducing or suppressing partially this competition. This is often done by mechanical or chemical methods. When living mulches are suppressed, the level of competition is decreased, but the soil surface is still protected. However, controlling the growth of the cover crop may not be always possible with mechanical mowing (Wilson et al., 1982).

Methods to study roots

General overview

Root systems are inherently difficult to study due to their underground environment, the complexity of the dynamic interactions with the environment, and the diverse type of functions root systems accomplish (Robinson, 1986). Destructive methods like core sampling of root mass (Böhm, 1979) are the most common approach that has been used in root studies; e.g. Pierret et al. (2005). But this method does not allow knowing the precise root position. A further main limitation of the latter approach is the impossibility to repeat the measurements for the same set of root systems. Non-destructive methods are more appropriate for the study of root interactions and especially root segregation since the temporal characteristics of these two processes make time one of the most important dimensions to take under consideration.

Observing the roots in rhizotrons with transparent windows interfaces, allows to follow the spatial and temporal distribution of root growth (Taylor et al., 1990), but this method has limitations because it is strongly disturbing the environment and compressing it almost into two dimensions. Minirhizotrons are transparent tubes inserted into the soil for the observation of root that belong to the category of non-destructive methods (Bates, 1937; Liedgens and Richner, 2001). They have the advantage to minimize the interference to root growth compared to the precedent method (Taylor, 1987).
General Introduction

Methods to study root interactions

With more than one species sharing the same soil, like for example in living mulches (Feil, 2001), interactions between the root systems will take place. Grace and Tilman (1990) qualify this interaction as a competition for shared and limited resources. Connell (1990) refers to it as an exploitative competition. However, there is few experimental data about root interactions due to the fact that roots as compared to the shoot grow in a rather inaccessible environment (Fitter, 2002) and to the lack of suitable methods to study these interactions (Hutchings et al., 2003). According to Liedgens et al. (2004b) it is impossible to distinguish the roots from maize and Italian ryegrass when grown in a living mulch system. This limitation prevents a better understanding of the cropping system and its further adoption by farmers. Only few studies exist for plant interactions at the root level between plants of two different species.

Pechakova et al. (1999) excavated the soil and tracked the roots to the plant they belonged to. Caldwell et al. (1996) combined a microscale root mapping approach and chemical technique to differentiate roots from a shrub and two species of grasses. They could show that at the microscale shrub and grass roots tended to avoid each other (i.e. segregated). According to Dudley and File (2007) the allocation of biomass to the root system of Cakile edentula increased when groups of foreign species shared a common pot, but not when the groups of plants were genetically related. These results demonstrate that plants can discriminate individuals in competitive interactions and indicate that the root interactions may provide the cue for this recognition. According to Gregory and Reddy (1982), trench-profile techniques can also be useful in observing interactions of root systems of different species in terms of spatial distribution. Radioisotopes can be incorporated into the plant and autoradiography used to study the distribution of roots in soil or the spatial relationships between the root systems of neighboring plants. These techniques are not used as widely as they were because of health and safety issues and because non-uniform distribution of label can make interpretation of results difficult. For example, when *Bouteloua gracilis* and *Gutierrezia sarothrae* were labelled with $^{14}$C and $^{86}$Rb, Milchunas et al. (1992) found that $^{14}$C activity was concentrated near the soil surface and $^{86}$Rb activity was highly variable and randomly distributed; neither technique produced the same
estimate of root distribution as excavation on nail boards. Jumpponen et al (2002) studied root distribution by relative uptake of tracers as $^{15}$N, $^2$H and $^{18}$O. Lehmann et al. (1998) used the discrimination of $\delta^{13}$C incorporation between C3 and C4 plant. It is also possible to use molecular tools based on the DNA and microsatellite analysis of root fragments (Brunner et al., 2001; Mommer et al., 2008).

In conclusion, up to now there is no reliable method available to determine the precise location of roots from different plants in space and time. Having such a method would allow understanding the belowground interaction of different plants in natural and managed ecosystems.
Hypotheses and objectives

The main hypothesis of this thesis is that a methodology that allows the study of spatial and temporal dynamics of coexisting root systems would be an important step forward towards the understanding of plant interactions in living mulch systems.

In consequence the first objective was to develop a method for the study of root interactions in mixed plant stands. The method was based on a maize genotype that had been previously transformed with a molecular marker, the green fluorescent protein (GFP). It was hypothesized that roots from this genotype would be exclusively visible in an inducing light. Therefore this fluorescent signal could be reliably traced in minirhizotron images and used to identify one of the plants in the mixed plant stand.

A further objective was to demonstrate that using maize transformed with the GFP combined with the adequate minirhizotron camera technique can allow distinguishing roots from different plants and studying their spatial and temporal distribution in a non destructive way.

The final objective was to study and characterize the spatial and temporal root distribution of maize and Italian ryegrass in a living mulch as an example of a mixed plant stand.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Abstract

The limited flexibility available in the configuration of commercial minirhizotron imaging systems makes it difficult to adapt these systems to new applications. It is also too expensive to introduce modifications, which are often very temporary, to these systems at the end of the development process.

In order to identify the roots of a single species in mixed plant stands, we developed a new minirhizotron imaging system that makes it possible to observe roots expressing green fluorescent protein (GFP). This system is based on affordable and easily obtainable components such as webcams. Here, we report a protocol to identify suitable webcams for constructing a minirhizotron imaging system and demonstrate the application of this protocol to build a minirhizotron imaging system that can identify the roots of a transformed maize plant expressing (GFP).

Keywords: roots, minirhizotron, webcam, imaging, green fluorescent protein.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Introduction

Minirhizotron imaging systems are a combination of light sources and small cameras constructed to fit into minirhizotrons (i.e., transparent tubes inserted into the soil). Minirhizotrons make it possible to study a broad array of biological processes such as: i) root development in the soil profile (Liedgens et al., 2000), ii) root turnover (Pregitzer et al., 2008), iii) root parasitism (Eizenberg et al., 2005), and iv) proliferation of fungal hyphae (Vargas and Allen, 2008).

Commercial minirhizotron imaging systems are available, but they have limitations such as: i) a relatively high price (>10,000), ii) rare updates incorporating recent technological progress due to low commercial demand, and iii) little flexibility in their configuration to enable the study of multiple processes with the same imaging system (e.g., root growth of multiple species in mixed plant stands or interactions with microorganisms).

Today, the widespread adoption of webcams could provide an opportunity to introduce faster, newer technology into minirhizotron research because they are affordable and are continuously updated to incorporate the latest developments in digital image-capturing technology. Many models include high-quality image sensors, and there is literature available on how to adapt them to different applications (e.g. Bendix et al., 2008; Faro et al., 2008; Gil et al., 2006; Janesick et al., 2002; Nedev and Ivanova, 2006; Ogren et al., 2004; Richardson et al., 2007; Sample, 2003).

The objectives of this study were the following: i) to develop a protocol to identify suitable webcams for constructing minirhizotron imaging devices and ii) to demonstrate the application of such a protocol to the building of an imaging system for identifying the roots of a transformed maize plant expressing green fluorescent protein (GFP).
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Materials and Methods

Components of a minirhizotron imaging system based on a webcam

Fig. 1 shows the principal components of a minirhizotron imaging system. These include: i) an image sensor, ii) optics, iii) a panel for the optics, iv) light, v) a case for the image sensor, vi) a handle.

Figure 1: Schematic representation of the components of a minirhizotron imaging system: Long pass filter (A). Band blocking filter (B). Panel for optics (C). Chassis to hold filter (D). Camera case (E). Light (F). Image sensor (G). Handle (H).
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Image sensor

Modern image sensors included in webcams are nowadays very efficient; in practice about 50% of the incoming light is captured, as compared to only 5% of the old pioneer systems (Blanksby and Loinaz, 2000). Two types of image sensors that are common nowadays: charge coupled devices (CCD) and complementary metal oxide semi-conductor (CMOS). Originally, CCD sensors had higher resolution and were more expensive. However, CMOS sensors had improved significantly in the last years and are nowadays at least as good as CCD for the proposed application in this paper (Bigas et al., 2006; Fischer, 2006; Litwiller, 2002; Litwiller, 2005).

There are two types of sizes to be considered when selecting an image sensor: i) the physical size of the sensor and ii) the image sensor grid size. In terms of the former, CMOS sensors have the additional advantage of being smaller for identical image resolution, allowing for further miniaturization. The physical size of the sensor is important as it has to fit inside the minirhizotron tube, determining the final size of the camera system. The image sensor grid size determines the resolution which can be achieved by the system. For the same image size a bigger grid size will allow a higher resolution. Image grid sizes of commercially available webcams are typically of 352 x 288 to 640 x 480 pixels. However, larger sensors are becoming available with grid sizes of 1280 x 1024 pixels. Conventional image sensors used for minirhizotron imaging have grid sizes of 640 x 480 pixels allowing for a resolution of about 60 μm for images of 18.0 x 13.5 mm (e.g. Bartz, Bartz Technology Co., Santa Barbara, CA, USA).

Optics

It is fundamental that the optics can focus the roots properly. Since the optics of many webcams are of bad quality (plastic) or missing (pin-holes), webcams that guarantee the quality of their optics must be chosen. For example, the QuickCam v11.5 by Logitech (Logitech International S.A., Romanel-sur-Morges, Switzerland) incorporates optics manufactured by Zeiss (Carl Zeiss AG, Oberkochen, Germany). An alternative may be achieved by ordering specific optics from specialized providers.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Panel for the optics

In order to make the minirhizotron imaging system more versatile and suitable for changing its configuration to different research objectives, we constructed a panel to hold different combinations of filters and lenses. Lenses and filters to excite or block light emissions of specific range can be attached to this panel to provide the imaging system a specific configuration.

Light

The dark environment in the minirhizotron makes artificial illumination necessary. A simple and affordable solution is to use LEDs (light emitting diode). Two LEDs (5500 K, Luxeon III Star, Philips Lumileds Lighting Company, San Jose, CA, USA) arranged around the optic are enough for obtaining images where roots and soil structures are visible. Strong light intensity has to be avoided since it causes undesired reflections at the minirhizotron interface. This issue was controlled by an assemblage of resistors to supply appropriate light intensity.

Case for the image sensor

The sensor’s location relative to the soil-minirhizotron interface can be rotated by 90° or placed in front of it, depending on the space available for the camera components inside the minirhizotron. The former allows for minimizing the space required, but it requires mirrors between the image sensors and the target objects. This makes the camera more complicated, expensive and difficult to maintain. As a result systems based on mirrors or that prevent the camera from being introduced in the minirhizotron were discarded a priori but could be considered for applications that demand very narrow minirhizotrons. A frontal localization has a simpler implementation as long as the space available for the image sensor and the optics is enough. For routine and safe operation, all components of the imaging system have to be assembled in a suitable case. During the development of the imaging system and for testing purposes we used a prototype made from a PVC tube with a smaller diameter than the
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

minirhizotron. However, for the later routine operation a more robust housing in metal was developed.

Handle

For the construction of a handle to hold the minirhizotron imaging system and to ensure proper image registration we followed a protocol reported elsewhere (Johnson and Meyer, 1998). This is a mechanical handle attached to the case for the image sensor that allows for introducing the system inside the minirhizotron and registering and collecting images. The handle couples a ratchet advancing mechanism with another mechanism that locks the handle to the minirhizotron tube, ensuring proper soil position registration. The handle allows moving easily the imaging system from one minirhizotron to another. Such a handle is commercialized as a separate component by Bartz (Bartz Technology Co., Santa Barbara, CA, USA).

Power supply

The camera is supplied with power via the USB port, which is also used to connect the camera to a computer, where images are digitally stored. The power of the USB port is not high enough to supply the lightning system. Currently an AC/DC power supply is used (Voltcraft Stecknetzgerät 3-12 V/1000 MA, Hirschau, Germany).

Software for the capture and analysis of images

Software to trigger the capture and name numerous images is available for free (http://videocapture.sourceforge.net/) or in commercial packages (http://www.bartztechnology.com/products.html). Once the digital images are acquired, image processing software of general characteristics (e.g. imageJ; http://rsbweb.nih.gov/ij/) or specifically developed to analyze minirhizotron images (http://www.ces.clemson.edu/~stb/rootfly/) can be used to determine the physical size of the pixels covered by roots in the images.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Comparison of webcams

We took a set of webcams available in the market and tested the quality of their images with an imaging test chart (Kodak Digital Science Imaging Test Chart TL-5003, 1995 Eastman Kodak Company). The set of cameras included: Quickcam pro 300 (Logitech International S.A., Romanel-sur-Morges, Switzerland), Zicplay TalkCam Tracer CCD (Zicplay S.A., St-Sulpice, Switzerland), Philips SPC 900 NC (Royal Philips Electronics Inc., Amsterdam, Netherlands), Creative livecam notebook pro (Creative Technology Limited, Singapore City, Singapore), and Logitech QuickCam express (Logitech International S.A., Romanel-sur-Morges, Switzerland).

We tested the webcams under two conditions: i) horizontal at a distance of 30 mm from the imaging test chart positioned flat and, ii) inside of a minirhizotron tube made of Plexiglas at 30 mm from the imaging test chart that was rolled over a minirhizotron. The cameras were compared on the basis of images of 640 x 480 pixels, with a resulting resolution of 40.6 μm.

Plant material

We tested the imaging system configured to identify roots of single species in mixed plant stands using a transgenic line of maize (Zea mays L.) expressing the GFP (Aulinger et al., 2003). The maize genotype ETH-M72 was genetically transformed to include the gene for the GFP (ETH-M72GFP). The transformation construct contains the gfp gene flanked by the ubiquitin promoter (ubi::gfp) and the NOS terminator and was cloned into the pUC19 vector, which contains the gene for ampicillin resistance (ampR) at the restriction sites SpeI and XbaI. The gfp gene was cloned into the cassette at the NcoI and SalI sites. The expressed GFP protein is reported to have a fluorescence peak between 500 and 520 nm when excited by light at 450 to 470 nm.

We grew the transgenic line of maize expressing GFP alone or together with the negative control (i.e., the non-transgenic maize line) in rectangular containers filled of soil (0.365 m x 0.265 m and 0.255 m) with two horizontal minirhizotrons at a soil depth of 0.10 m. Eight plants per box were sown. We took pictures using the minirhizotron imaging system with the conventional configuration and that to identify roots of single species in mixed plant stands.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

**Results and discussion**

*Development of a minirhizotron imaging system*

The first step was the identification of suitable webcams in the market. Fig. 2 shows the results of the imaging test.

<table>
<thead>
<tr>
<th>Webcam</th>
<th>Flat</th>
<th>In Tubes</th>
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<tbody>
<tr>
<td>Quickcam pro 300</td>
<td><img src="image1" alt="Flat Image" /></td>
<td><img src="image2" alt="In Tubes Image" /></td>
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<tr>
<td>Zic play, TalkCam Tracer CCD</td>
<td><img src="image3" alt="Flat Image" /></td>
<td><img src="image4" alt="In Tubes Image" /></td>
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<tr>
<td>Philips SPC 900 NC</td>
<td><img src="image5" alt="Flat Image" /></td>
<td><img src="image6" alt="In Tubes Image" /></td>
</tr>
<tr>
<td>Creative, livecam notebook pro</td>
<td><img src="image7" alt="Flat Image" /></td>
<td><img src="image8" alt="In Tubes Image" /></td>
</tr>
<tr>
<td>Logitech QuickCam express</td>
<td><img src="image9" alt="Flat Image" /></td>
<td><img src="image10" alt="In Tubes Image" /></td>
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*Figure 2: Webcams imaging test*
This test measures the imaging characteristics of webcams based on the following quality parameters: i) aspect ratio, ii) geometric distortion, iii) exposure uniformity, and iv) light uniformity. By this test it was possible to discard those webcams that produced images with distortions like Quickcam pro 300 and Zic play (Fig. 2). A set of webcams that produced images of acceptable quality remained. The imaging test also provided information to decide whether was better to use the build in optics or those from a specialized provider. Using optics supplied by an external provider was against the objective of building an affordable camera. Therefore livecam notebook pro, manufactured by Creative, was the right choice. This was a good compromise, too, among a camera size small enough to be included in a tube, the area of the captured images, and the quality of the images. The 640 by 480 pixels images cover an area of 26.0 x 19.5 mm, with a resulting resolution of 40.6 μm. However, any of the other two webcams tested that had satisfactory quality parameters would have probably been also suitable for developing a minirhizotron imaging system.

The second step was to identify the settings of the other components of the imaging system. This must be done in a specific manner according to the webcam that was selected. The relatively small size of the webcam that we chose allowed for assembling the image captor directly under the target area of the images. For calibrating the webcam, it was necessary to determine the physical size of the images captured by placing a graduated millimeter ruler over the captor.

Figures 3a and 3c show images obtained with the conventional minirhizotron imaging system; roots and soil pores are clearly visible which demonstrates the high resolution of these images. These images are of similar quality as those from commercial minirhizotron cameras and fulfill the requirements of software to analyze root images (e.g. rootfly). Therefore, they demonstrate that constructing a minirhizotron imaging system using webcams is possible. Furthermore, following the steps described here, the resolution can be increased as well as the light characteristics to satisfy specific research goals.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

Figure 3: Minirhizotron images obtained with the conventional imaging systems (a, c) and with the imaging system where the expression of the green fluorescent protein can be detected (b, d). The images were taken for non transgenic maize (a, b) and for maize expressing the green fluorescent protein (c, d).

Adaptation of the imaging system to identify fluorescent roots

Departing from the conventional minirhizotron imaging system described above, we developed an imaging system for identifying roots that can emit green fluorescence; for this purpose the transgenic ETH-M72GFP was used. The feasibility of the proposed approach was inferred for images taken with a microscope, showing that under normal light roots of plants expressing or not the gfp protein are indistinguishable from each other, while, with proper lightning and filtering, only roots of plants expressing the gfp protein are visible. This system was based on the conventional minirhizotron imaging system except for a different set of lights and three additional filters attached to the panel for the optics.
Chapter 1: A minirhizotron imaging system to identify roots of single species in mixed plant stands.

The detection of the roots of ETH-M72\textsubscript{GFP} depends on the excitation of the protein by supplying light in the appropriate range. In microscopy Hg light is typically used. This is, however, no practical with minirhizotrons since the heat that they produce damage the minirhizotron tubes. Because LEDs that emit light at wave lengths required to excite the protein exist, we used LEDs that covered the narrow light spectrum of 440 to 460nm (Luxeon V Star, Philips Lumileds Lighting Company, San Jose, CA, USA).

Upon correct activation of the \textit{gfp} protein, fluorescence should be visible with an adequate set of filters. On selecting such filters for an affordable imaging system it was important to find a compromise between price and quality. Cheap plastic filters did not provide satisfactory blocking of the incoming light spectrum, therefore more specific glass filters had to be chosen. A band blocking filter was used to affine the wavelength window of the light (Dichroic 555 IM 25, Comar Instruments, Cambridge, UK). A longpass color glass filter (LONG 515 nm, Edmund Optics, Barrington, USA) was installed in front of the image captor to block the wavelengths outside the fluorescence spectrum of the GFP. This precise adjustment of the light source and the captured light was important to avoid the detection of auto-fluoresce from roots and elements in the rhizosphere.

Figures 3b and 3d shows images obtained with the minirhizotron imaging system modified to detect roots of ETH-M72\textsubscript{GFP}. In these images the roots of ETH-M72\textsubscript{GFP} are visible and it is easier to distinguish roots from the background in these images (Fig. 3b and 3d) than in conventional images (Fig. 3a and 3c) because of the better contrast. Therefore, the basic configuration of the minirhizotron imaging system based on a webcam could be easily adapted to detect the fluorescent roots of ETH-M72\textsubscript{GFP} and overcome the problem of the low flexibility in the construction of commercial equipments. This low flexibility makes difficult and costly to conduct the tests that are necessary to extend the applications of minirhizotrons. The cost of the imaging systems that we reported here is of $195 for the conventional images and $390 for the images where fluorescent roots are visible.
Conclusion

Here we demonstrate the feasibility of developing an affordable minirhizotron imaging system for capturing conventional images and for images where fluorescent roots are visible. This was necessary to overcome the low flexibility in the configuration of commercial systems and thus extend the application of minirhizotrons. The system to detect roots of ETH-M72\textsubscript{GFP} constitutes the first report of an imaging system to identify roots of single species in mixed plant stands.

Furthermore, the steps proposed here can be implemented to develop and configure imaging systems to extent the applications of minirhizotrons. This could be the case for devices to screen rhizotrons or to capture images of high resolution where mycelium of ectomycorrhiza is visible. There are currently no imaging devices to conduct the latter two tasks.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

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Faget, M., Herrera, J.M., Stamp, P., Aulinger-Leipner, I., Frossard, E., and Liedgens, M. 
*The use of green fluorescent protein as a tool to identify roots in mixed plant stands.*

**Abstract**

Although roots take up most of the resources required by the plant, a lack of efficient research tools hinders our understanding of the function and relevance of the root system. This is even more evident when the research focus is not on a single plant, but on multiple plants that share the same soil resources. None of the available methods allow for simple, inexpensive, non-destructive, and objective assignment of observed roots in a mixture of plants to a target plant. Here, we demonstrate that transgenic plants expressing the green fluorescent protein (GFP) combined with the well-established minirhizotron technique are a route to overcoming this limitation. We planted transgenic maize (*Zea mays* L.) in combination with either its corresponding wild-type, Italian ryegrass (*Lolium multiflorum* Lam.) or soybean (*Glycine max* (L.) Merr.). The identification of fluorescent roots allows the relative distribution of roots of each plant type and their interaction and interference with each other to be observed. The selected plants are suitable for model experiments to unravel fundamental belowground ecological processes. Because genetic transformation of plants is an established technique that can be applied to a large set of plant species, this method will be of interest to a broad range of research areas.

**Keywords:** root research methodology, root interactions, green fluorescent protein (GFP), mixed plant stands, minirhizotron, and imaging system.
Introduction

Roots are essential for plant growth, survival, and fitness. Plant species differ in their temporal and spatial exploration of the soil and in their adaptation to biotic and abiotic stresses. Due to the heterogeneous nature of soil environments, root systems must respond to a wide spectrum of physical, biological, and chemical conditions, including resource availability, all of which show spatial and temporal variation. Consequently, root systems develop into a complex array of irregularly distributed roots. These root arrangements ultimately determine the ability of the plant to access soil resources (Robinson, 1994).

To determine the distribution of roots in the soil, a diverse set of approaches was employed. Destructive sampling of roots has been the research method of choice for more than a century. The most common approach is to sample soil cores, which are small compared to the rooting volume (Pierret et al., 2005). However, with these methods it is impossible to determine the exact position of the roots. Root position is important because resource uptake is usually affected to a greater extent by the relative spatial distribution of roots and soil resources than by the size of the root system (de Kroon, 2007). Also, these methods do not enable multiple measurements of the same roots. Non-destructive screening by means of transparent observation interfaces overcomes this limitation and is effective for following the spatial and temporal dynamics of root growth (Taylor et al., 1990). A disadvantage of this method is that it modifies the rooting environment. However, the degree of interference is much smaller for minirhizotrons (i.e., transparent tubes inserted into the soil) compared to classical rhizotrons (Taylor et al., 1990). Despite the abovementioned approaches, a simple, inexpensive method to assess the distribution of roots in the soil does not exist.

Root systems usually share the soil with intraspecific or interspecific neighbours. Thus, the relative arrangement of the roots will influence processes such as resource capture and root exudation (Li et al., 2007). However, the methodological constraints to assess root distribution are even more acute when the focus is on the coexistence of multiple plants, i.e., when they share the same soil volume, rather than on single plants. For such cases, it is also necessary to
determine which root belongs to which plant. This is very difficult to be done visually on a high number of samples since roots as opposed to flowers or leaves show few distinctive external features that would permit identification of species (Fitter, 2002).

The relative distribution of roots of different plant species was studied by the following methods: (i) the relative uptake of tracers such as $^{15}$N, $^2$H, and $^{18}$O (e.g. Jumpponen et al., 2002); (ii) $\delta^{13}$C discrimination between C$_3$ and C$_4$ plants (Lehmann et al., 1998); (iii) labelling the shoots of each species with different radioactive tracers in order to distinguish their roots from the differential signature on autoradiographs (Baldwin and Tinker, 1972); (iv) excavating the soil and tracing roots back to the plant to which they belong (Pechackova et al., 1999); (v) mapping all the roots at the cut surface of a core and conducting a chemical extraction to distinguish roots of different species according to the colour and fluorescence intensity of the resulting eluant (Caldwell et al., 1996); and (vi) using molecular tools based on the extraction of DNA and microsatellite analysis of root fragments (Brunner et al., 2001). None of these approaches has become a standard investigation tool, and, except for the method used by Caldwell et al. (1996), they do not allow the determination of the relative location of roots belonging to different plants. However, such information is important for understanding and managing the coexistence of plants in natural and managed ecosystems (de Kroon, 2007; Schenk, 2006).

Fluorescence is widely applied in the natural sciences as a visual reporter for localisation purposes. For example, autofluorescence has been used to report the colonisation of roots by microorganisms (Gamalero et al., 2003), and staining with fluorescent substances has been used to analyse plant structures (Lux et al., 2004). The use of fluorescent proteins was such an impressive methodological achievement for research that it was awarded the Nobel Prize for Chemistry in 2008. By direct or associated tagging, fluorescent proteins allow for a plethora of scientific applications aimed at the temporal and spatial monitoring of processes in living cells and organisms (Chalfie and Kain, 2006).
We genetically transformed the maize genotype ETH-M72 to include the gfp gene for the green fluorescent protein (GFP) from the jellyfish Aequorea victoria (Aulinger et al., 2003). Here, we demonstrate that fluorescent roots are a powerful tool for investigating the relative distribution of roots, especially fine roots, belonging to different plant species, a new experimental approach in functional root ecology. Furthermore, we show that minirhizotrons enable continuous and non-destructive observation of the fluorescence expression of GFP maize. The plant set used for this study includes the genetically transformed maize genotype, its corresponding wild-type, Italian ryegrass, and soybean.

Material and methods

Plant material

The maize genotype ETH-M72 was genetically transformed to include the gene for GFP (ETH-M72\textsubscript{GFP}). The transformation construct contains the gfp gene flanked by the ubiquitin promoter (ubi::gfp) and the NOS terminator. It was cloned into the pUC19 vector, which contains the gene for ampicillin resistance (ampR) at the restriction sites SpeI and XbaI. The gfp gene was cloned into the cassette at the Ncol and SalI sites. The expressed GFP is reported to have a fluorescence peak between 500 and 520 nm when excited by light at 450 to 470 nm. Validation of the genetic transformation, the integration pattern of the inserted gene, and comparisons of the homozygosity of transgenic and non-transgenic controls are reported in Aulinger \textit{et al.} (2003).

The ETH-M72\textsubscript{GFP} maize was grown together with either the untransformed wild-type (ETH-M72\textsubscript{WT}), Italian ryegrass (Lolium multiflorum Lam., cv. Ellie), or soybean (Glycine max Merill, cv. Amphor). The arrangement with ETH-M72\textsubscript{WT} and with non-maize plants represents monocrop and intercropping systems, respectively.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

Experimental conditions

The data presented herein were collected from two sets of experiments in which plants were grown either in pouches without soil or in large containers with soil. Maize $\text{ETH-M72}_{\text{GFP}}$ was grown alone, with wild-type maize and with the other plant species.

Germinated seeds of $\text{ETH-M72}_{\text{GFP}}$ ($n=30$) and $\text{ETH-M72}_{\text{WT}}$ ($n=30$) maize were transferred alone or together to moistened blotting paper (21×29.5 cm; Anchor Paper, St. Paul, MN, USA) in pouches (Hund et al., in press). The pouches were hung in plastic containers (27×37×32 cm; Arcawa GmbH, Chatillon, Switzerland) in order to submerge the bottom 20 mm of the blotting paper in water. To avoid a light impact on root growth, the pouches were covered with a 0.5-mm thick black polyethylene foil (PE-Teichfolie Typ WA-1200, Walser Kunststoffwerk AG, Buerglen, Switzerland). In addition, to avoid heating the pouches, the containers were covered with aluminium foil, except for a narrow opening through which the seedling grew. The plants were grown for 7 days in a growth chamber (PGW36 Conviron, Winnipeg, MB, Canada) at a temperature of 24°C, a relative humidity of 70%, a photosynthetic active radiation of 400 $\mu$mol sec$^{-1}$ m$^{-2}$, and a photoperiod of 16 h. The blotting papers with the plants were removed from the pouches to acquire images of the roots.

For the experiments with soil, $\text{ETH-M72}_{\text{GFP}}$ maize was grown alone and together with Italian ryegrass in large containers, i.e., wooden boxes (0.60 m wide x 0.80 m long and 0.80 m high), allowing for unrestricted root growth. Minirhizotrons (54 mm inner diameter, 60 mm outer diameter) were installed horizontally in these wooden boxes at a depth of 0.15 m. Minirhizotrons were in the middle of the boxes, at 0.30 m from the edges. Italian ryegrass was sown at a density of 6.27 g seed per m$^2$. After 40 days, a 0.30-m wide strip of Italian ryegrass in the mid-section of the boxes and perpendicular to the longer side was cut and removed from the box. Within that strip, $\text{ETH-M72}_{\text{GFP}}$ maize plants were sown at a density of 10.4 plants per m$^2$. The orientation of the resulting maize row was perpendicular to the minirhizotrons. Thereafter, the remaining grass plants were cut to 0.15 m at weekly intervals. The mean temperature in the greenhouse was 23°C, and the photoperiod was 16 hours. The water content of the soil was maintained by irrigation, as estimated by a time domain reflectometry probe (EC-5 Soil Moisture Sensor, Decagon Devices...
Inc., Pullman, WA, USA). The experiment was conducted until the anthesis of maize, which occurred approximately at 83 days after sowing.

ETH-M72GFP maize was also grown alone and together with either ETH-M72WT or soybean in circular PVC containers (0.60 m inner diameter x 0.70 m high) with a single horizontal minirhizotron at a depth of 0.20 m, in the same greenhouse environment as the experiment in rectangular wooden containers. Ten ETH-M72GFP maize plants in the containers with ETH-M72GFP alone, and five plants of each type in the containers with two types of plants were sown at the same time. The containers with two types of plants were separated into two equal parts perpendicular to the orientation of the minirhizotrons, to which one of each plant type was assigned. Plants within a single half of each container were arranged in a regular pattern. The minimum distance between two plants of different types was 0.12 m.

The substrate in all containers was a mix of loamy field soil and sand and had according to the method given in parentheses the following characteristics: 30 g kg\(^{-1}\) organic matter (Blake-Walkley), 60 g kg\(^{-1}\) clay, 110 g kg\(^{-1}\) silt, 810 g kg\(^{-1}\) sand, pH 7.7, 66.3 mg kg\(^{-1}\) assimilable K (NH\(_4\) acetate), 4.1 mg kg\(^{-1}\) P (Olsen), and 12 g kg\(^{-1}\) N (2M KCl). The wooden boxes and the PVC containers were filled three months before starting the experiments to enable the soil to settle. Foskal\(^{\circledR}\) (P\(_2\)O\(_5\), K\(_2\)O, Mg, Ca, and S; Agroline, Basel, Switzerland) and ammonium nitrate (NH\(_4\) NO\(_3\)) were applied before sowing to supply 1.4 g P, 8 g K, 0.57 g Mg, 0.20 g Ca, 0.87 g S, and 1.1 g N per m\(^2\).

**Imaging equipment**

Although there is commercial equipment available for imaging the soil-minirhizotron interface (e.g., Bartz Technology Company, Carpinteria, CA, USA), its configuration does not allow for the visualisation of fluorescence. However, it is possible to construct imaging systems to test the suitability of the fluorescence method, as devices for capturing images (such as web cameras) are widespread and inexpensive. Therefore, we selected a web camera (Live Cam Notebook Pro\(^{\circledR}\), Creative Technology Limited, Singapore City, Singapore) to digitally capture minirhizotron images. The unmodified camera, one of the smallest currently available, was
integrated into a very simple assembly (Fig. 1a) with additional light emission devices (LED). Its basic configuration did not deviate fundamentally from the design proposed by Upchurch and Ritchie (1983). The 640 x 480 pixel images cover an area of 26.0 x 19.5 mm, with a resulting resolution of 40.6 μm. The LEDs were connected to an external power supply (Volcraft Stecknetzgerät 3-12 V/1000 MA, Hirschau, Germany), and the camera was powered by the USB computer interface. Based on this basic module, two configurations were constructed (Fig. 1a and 1b). One configuration included white LEDs (5500 K, Luxeon III Star, Philips Lumileds Lighting Company, San Jose, CA, USA) for the sampling of images to display all structures at the interface of the minirhizotron. These images will be referred to as “conventional images”. The second configuration differed from the first one by: (i) LEDs covered a narrower light spectrum of 440 to 460 nm (Luxeon V Star, Philips Lumileds Lighting Company, San Jose, CA, USA) close to the GFP excitation range; (ii) a band-blocking filter was used to avoid sources of green light (Dichroic 555 IM 25, Comar Instruments, Cambridge, UK); and (iii) a long-pass colour glass filter (LONG 515 nm, Edmund Optics, Barrington, USA) was installed in front of the webcam to block as many of the long light waves outside the fluorescence spectrum of GFP as possible. This precise adjustment of the light source and the captured light is important to avoid the detection of auto-fluorescing substances from the roots and the rhizosphere. Images sampled by the latter configuration display the fluorescent roots of ETH-M72GFP and are referred to as “fluorescent images”. The assemblies used to capture the images described above were mounted on the tip of a handle similar to the one described by Ferguson and Smucker (1989). This guarantees that the same position at the minirhizotron interface may be imaged repeatedly and precisely with both configurations. The use of identical handles for both cameras enabled us to repeatedly sample images of both types at the same position in the minirhizotrons over the entire duration of the experiment. Conventional minirhizotron images and fluorescent images were obtained from the blotting paper with roots and from the upper surfaces of the soil-minirhizotron interface. The minirhizotron images covered a strip of 19.5 x 312 mm (i.e. 12 positions) in both experiments with soil. Images for the experiments in pouches were obtained 7 days after germination, while those for the soil experiments were obtained up to 48 (for ETH-M72GFP and Italian ryegrass) or 72 (for ETH-M72GFP, ETH-M72WT and, soybean) days after sowing the maize plants.
**Screening of roots growing in soil**

The number of roots (i.e. number of root incidences on the minirhizotron interface) and root length for each of the sampled images was determined according to Upchurch and Ritchie (1983) and Atkinson (2000), respectively. Although these estimates can be obtained from single minirhizotron images, the accuracy of identifying root structures in images increases when images acquired at the same position are captured across a set of sampling dates (Smit et al., 2000). Therefore, all images recorded at a single minirhizotron position over the entire growing season were organised into an image time series (ITS). Since no objective visual criteria exist to determine whether a root is functional (Smit et al., 2000), all roots identified in an image were accounted for in each ITS. Thus, the obtained response variables were cumulative no. of roots and cumulative root length (cm cm\(^{-2}\)) observed per image since the sowing date. These were converted into a surface unit (cm\(^2\)) and are referred to hereafter as root density (roots cm\(^{-2}\)) and root length density (cm cm\(^{-2}\)), respectively.

The image set (n=3200) used to validate the proposed method consisted of 100 ITS, each including images sampled on 16 dates, randomly selected from plots containing only ETH-M72\(_{GFP}\). Conventional and fluorescent images were screened for each ITS. Images without roots were included to account for false positives (i.e., identification of non-root objects as roots).

**Statistical analysis of the effect of image type on root parameters**

Effects of the type of image, i.e., conventional or fluorescent, on estimates of root density and root length density of ETH-M72\(_{GFP}\) were tested using analysis of variance. Image type, plot, and the interaction between image type and plot were considered as factors in the statistical model. Deviations from the 1:1 relationship between conventional and fluorescent images were studied by taking the values from conventional images as the reference (expected values) and comparing them those in fluorescent images (observed values). The analysis of variance and the deviation from the 1:1 relationship for root density and root length density between the values obtained from fluorescent images and those from conventional images were performed for the last image of the ITS using the functions aov() and lm() in the statistical software R, respectively.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

Results

Expression of GFP along ETH-M72GFP roots in pouches

Images of roots obtained from pouches are shown in Figure 1 (c to h) as examples of the type of results obtained with fluorescing roots. Images c, e, and g depict conventional images, whereas images d, f, and h depict fluorescent images. The latter images show that although roots from ETH-M72WT could sometimes be noted in fluorescent images (Fig. 1d), the expression of GFP in the ETH-M72GFP results in roots with green light emitted (Fig. 1f) that is not observable for ETH-M72WT (Fig. 1d). Furthermore, we did not observe ETH-M72GFP roots that lacked this fluorescent intensity. As a result, roots from ETH-M72WT and ETH-M72GFP are indistinguishable in conventional images (Fig. 1g), but clearly distinguishable in fluorescent images (Fig. 1h). Therefore, it is possible to conclude that: (i) roots showing bioluminescence belong to ETH-M72GFP plants (Fig. 1h), and (ii) the GFP expression and thus fluorescence is observable throughout the extension of the main root axis and its branches (Fig. 1f and 1h).

Root screening of ETH-M72GFP grown alone in the soil

The image set used to validate the proposed approach consisted of 100 ITS obtained from plots in which ETH-M72GFP was grown alone. The same positions at the soil-minirhizotron interface were screened independently using either conventional or fluorescent images and the results obtained from each type of image were compared. For single ITS’, root density from the fluorescent images varied between +52% and -28%, compared to the conventional images. Similarly, for root length density, these differences ranged between +62% and -10%. On average, root density and root length density for the ITS were recorded as 28% and 16% higher in fluorescent images compared with conventional minirhizotron images. Figure 2a and 2b show the relationships between values obtained from fluorescent images and those from conventional minirhizotron images for root density and root length density, respectively.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

**Figure 1:** Camera used to sample conventional images (A). Camera used to sample fluorescent images (B). Adapted Webcam (I). White LEDs (II). Blue LEDs to excite the GFP (III). Filters used to restrict the light to the range of GFP excitation (IV). Filter used to restrict light from being captured by the image sensor for GFP fluorescence (V). Roots of the maize (*Zea mays* L.) genotype ETH-M72<sub>WT</sub> (C), ETH-M72<sub>GFP</sub> (E), and both together (G) growing in pouches as visible in conventional (C, E, and G) and fluorescent images (D, F, and H).
Figure 2: Relationship of the root densities (A) and root length densities (B) measured with fluorescent and conventional images. The line indicates the 1:1 ratio. The box diagrams in each plot show the distribution of the values obtained by subtracting the results of conventional images from those of fluorescent images.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

It was significantly different from the 1:1 relationship for root density (F-value=9.35, p-value=0.002, d.f.=194) and for root length density (F-value=17.0, p-value<0.001, d.f.=194) and it was not linear, with systematic deviations from the estimated line identified for larger values. Also the median differences between the estimates of the root parameters for both image types (box diagrams) were higher than 0, indicating that the values were skewed towards high values. The differences in fluorescent compared to conventional images were especially high for root densities over 3 roots cm\(^{-2}\) and root length densities over 2 cm cm\(^{-2}\). Box diagrams also showed that, except for some potential outliers, the differences between the estimates for both image types were approximately normally distributed, and the distribution of the differences among root length densities were more skewed than the distribution of the differences among root densities. An analysis of variance revealed marginal effects from the type of image used to measure root density (F-value = 3.22, p-value = 0.07, d.f. = 197) and insignificant effects for root length density (F-value = 1.39, p-value = 0.24, d.f. = 197).

Larger values in fluorescent images compared with conventional images often resulted from the fact that some of the roots were only observed in the fluorescent images (Fig. 3d and 3l) because of the improved contrast between the roots and the background in those images. In conventional images, transparent roots may be overlooked and some roots are very difficult to distinguish from other image structures. This is not a factor for the ETH-M72\(_{GFP}\) roots in fluorescent images. Similarly, in fluorescent images, two or more root segments that are closely located to other root segments are more easily recognised as separate root segments. Lower values for the fluorescent images can arise from multiple root segments not being recognised as a single unit; i.e., the integrity of the root segment is lost due to poor contrast with the background.

In conventional images, these segments are interpreted as separated root segments according to the rule proposed by Upchurch and Ritchie (1983). Furthermore, lower values for fluorescent images compared to conventional images also denote the risk in conventional images due to the uniform background, to erroneously identify image structures as roots when they are not. In fluorescent images this is not a factor. Nevertheless, the fact that higher values for both root density and root length density are more common in fluorescent images shows that
underestimation rather than overestimation is the more serious source of error when screening roots with conventional minirhizotron images. Similarly to the pouches experiment, we did not observe any ETH-M72$_{\text{GFP}}$ roots that lacked green light emitted at any point during the screened sequence from the minirhizotrons inserted in the soil. This observation was expected because the \textit{gfp} gene is constitutively expressed. These results confirm that all ETH-M72$_{\text{GFP}}$ roots can be identified in fluorescent images obtained from minirhizotrons inserted in the soil.

\textit{Discrimination of roots between interspecific neighbours}

For each of the two combinations of plants studied in containers with soil, one set of four pictures is shown (Fig. 3). Images a to d and e to h depict roots of Italian ryegrass and soybean together with ETH-M72$_{\text{GFP}}$, respectively. The images depict conventional and fluorescent images sampled at two different dates. All of the conventional images include structures that can be identified as roots, but other elements, such as soil particles and voids, are also visible.

From the conventional images of roots, it was not possible to assign roots to the plant type. The fluorescent images proved that only roots of Italian ryegrass, soybean, and ETH-M72$_{\text{WT}}$ exist at the sampled location on the first sampling date (Fig. 3), whereas ETH-M72$_{\text{GFP}}$ roots (white arrows) coexist with roots of the companion plants on the second sampling date. The fluorescence of the meristematic tissues, such as the tips and branch insertions of ETH-M72$_{\text{GFP}}$, were particularly bright. Some ETH-M72$_{\text{GFP}}$ roots were visible only in the fluorescent images.

Structures in the conventional images are the sole result of light reflection. Roots must fluoresce within the range defined by the filter (515 nm) installed in front of the imaging sensor in order to be detected in the fluorescent images (Fig. 1b). The low amount of light detected by the image sensor originates from reflection because the wavelength range of the LEDs (440 to 460 nm, Fig. 1b) and the fluorescence do not overlap. The identifiable roots in both types of images are of one of three types: those that are only visible in the conventional images, those that are visible in the conventional and in the fluorescent images, and those that are only visible in the fluorescent images. The roots in the conventional images have no characteristics that would enable them to be assigned to one of the plants in the group. Since the green fluorescence of the
ETH-M72\textsubscript{GFP} roots is the consequence of the expression of a protein naturally absent in plants, it is concluded that roots visible in the fluorescent images belong to ETH-M72\textsubscript{GFP}. In the images obtained for containers with ETH-M72\textsubscript{GFP} plants only, roots observed in conventional images were also visible in fluorescent images.

In addition, some ETH-M72\textsubscript{GFP} roots were visible only in the fluorescent images (Fig. 3d and 3l). These are typically fine roots that, as found for containers with ETH-M72\textsubscript{GFP} only, are not identifiable in the corresponding conventional images (Fig. 3c and 3k) because they are either too fine or are transparent. Roots that lack green light emitted, as shown in the pouches experiment (Fig. 1c to 1h), belong to companion plants rather than to the ETH-M72\textsubscript{GFP} plants.
Figure 3: Minirhizotron images of mixed plant stands with the genetically transformed maize (*Zea mays* L.) genotype ETH-M72<sub>GFP</sub> and one of the following: Italian ryegrass (*Lolium multiflorum* Lam., A to D), soybean (*Glycine max* Merill, E to H), or the maize genotype ETH-M72<sub>WT</sub> (I to L).

The conventional minirhizotron images in the left-hand column were captured within the visible light spectrum, 37 (A), 48 (C), 65 (E), 72 (G), 47 (I), and 58 (K) days after sowing the maize. The fluorescent images in the right-hand column were captured with a narrow-spectrum light source to trigger fluorescence on the same days as the corresponding conventional images. From the conventional images of the roots, it was not possible to assign roots to the plant type.

Roots of Italian ryegrass (black arrows, A), soybean (red arrows, E), ETH-M72<sub>WT</sub> (blue arrows, I), and ETH-M72<sub>GFP</sub> (white arrows) can be recognised. Tips (○) and branch insertions (□) of the ETH-M72<sub>GFP</sub> roots (D) were particularly bright in the fluorescent images (L).

Some maize roots were visible in the fluorescent images only, i.e., D and L (Δ). Scale: 1 pixel = 40.6 μm.
Discussion

This is the first report to demonstrate the use of fluorescence resulting from GFP expression to study the interactions and interference of roots in mixed plant communities. It presents a method that allows for the distinction of roots belonging to different plants that are grown together. This method is based on the use of genetically modified plants in which the constitutive expression of GFP results in a bioluminescence throughout the root extension (Fig. 1f and 1h). This green emitted light is not observed in non-genetically transformed maize, soybean, and Italian ryegrass. The fluorescence of the ETH-M72<sub>GFP</sub> roots can be used as a visual marker to quantify the root density and root length density of intraspecific and interspecific neighbours.

Although this method requires at least one transgenic fluorescent plant species, it can finally replace methods that rely on the separation of rooting volumes by barriers (Li et al., 2007) or trenches (Ludwig et al., 2004). Such methods drastically disturb the soil environment and, in extreme situations, can lead to an artificially-induced response in the plant (Connell, 1990). However, Campbell (1994), for example, has reported to have distinguished wild cherry (<i>Prunus avium</i> L.) from grass roots by their generally thicker diameter, opacity, more succulent appearance, and colour. The lack of further reports applying these criteria for distinguishing roots indicates the difficulty in adapting the procedure to a larger range of plant species combinations. Furthermore, for universal applications, a new methodology for discriminating between root systems should allow for sampling of a sufficiently large numbers of roots, as required for a representative estimation of fine root traits (Pierret et al., 2005). This new method meets these requirements and will provide the urgently needed experimental evidence of the relative distribution of root systems in the soil on the condition that at least one of the desired genotypes or plant partners in the mixture can be genetically transformed for experimental purposes.

This method allows for a very fine-scaled resolution in time and space, which is not the case for other proposed approaches. Tracers (e.g. Jumpponen et al., 2002) and $^{13}$C discrimination (Lehmann et al., 1998) only allow for coarse temporal and spatial resolution due to the expense of the tracers and of their chemical analysis. Furthermore, the relative arrangement of fine roots
cannot be determined. Although these methods enable estimates of the root mass fractions, the distribution of roots is more suitable for measuring resource capture (Atkinson, 2000). Root distribution can be precisely generated by this proposed method by delivering data for the root arrangement and enabling the measurement of other root traits, e.g., diameter, branching, turnover, and vitality.

In addition to the original goal of discriminating roots, this method also represents a means of increasing the accuracy and precision of minirhizotron assessments because errors in estimating root parameters can be reduced by using GFP as a visual marker. Analysis of variance did not reveal a significant effect of the image type on the measured root parameters. Therefore, to compare treatments, both types of images will likely result in similar conclusions. However, root fluorescence can improve the capacity of researchers to precisely estimate root parameters, information which is critical to generate input data in the context of model simulations. For example, Robinson (2007) estimated that the imprecise quantification of root biomass is responsible for 68% of the underestimation of the root carbon pool commonly assumed for global carbon inventories. We found that root density and root length density were on average +28% and +16% higher using fluorescent images than using conventional images.

An interesting method based on molecular diagnostics has been presented for precise distinction of roots in combinations of plants (Brunner et al., 2001; Mommer et al., 2008). The method based on molecular diagnostics is a powerful tool because if molecular markers for the species exist, it has the capacity to distinguish the roots of a single plant and to assign all roots in the sample to a certain species. Due to the necessity for destructive sampling and sample cost, this molecular method would be more suitable for studies focusing on static rather than dynamic characteristics of coexisting roots. In contrast, the GFP-based method focuses on the dynamic analysis of coexisting root systems.

It may be interesting to use the fluorescence method with destructive (auger) sampling as well. This is theoretically possible, and simply depends on the construction of imaging devices with lights and filters suitable for detection of fluorescence in the collected samples. However, the persistence of the fluorescence after destructive sampling may limit the suitability of this
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

approach for large numbers of samples. Additional drawbacks that may be relevant when considering this method are: (i) it has been reported that there is no GFP expression under anaerobic conditions (Chalfie and Kain, 2006), preventing its use in flooded soils, and (ii) it is based on genetically modified plants, which may restrict its application for open air experiments in several countries.

Compared to all of the other methods proposed to study the relative distribution of roots in plant mixtures, we believe that observation of the root fluorescence of genetically transformed plants through minirhizotrons is currently the simplest method for the large number of samples necessary for reliable statistical inference. Transgenic constructs expressing fluorescent proteins in colours other than green are available, opening up new avenues for studying complex belowground plant interactions with more than two plant species. Such a study would be especially interesting for natural or managed ecosystems, provided that transgenic experimental plant types can be obtained.
Chapter 2: The use of green fluorescent protein (GFP) as a tool to identify roots in mixed plant stands

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Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Abstract

The use of living mulches for the cultivation of maize (*Zea mays* L.) may reduce soil erosion and nitrate leaching. The grain yield of maize in such environmentally sound cropping systems is often low, however. We recently developed an innovative method that allows for the direct and non-destructive observation of the root growth of maize cropped in a living mulch system. This method is based on the use of transgenic maize expressing the green fluorescent protein (GFP) and minirhizotrons. In the present study, transgenic maize expressing GFP was grown as sole crop on bare soil or together with living Italian ryegrass (*Lolium multiflorum* Lam.) in boxes (0.48 m² surface area x 0.80 m depth) in a greenhouse. Minirhizotrons (60 mm outer diameter) were installed horizontally and perpendicular to the maize row at three soil depths. Root growth was screened from sowing to the onset of anthesis of maize. Compared to maize cultivated on bare soil, the living mulch reduced the shoot dry matter of maize at anthesis by 56%. The reductions in leaf area (39%) and average root density (41%) were almost identical. At maize anthesis, maize roots constituted 32% (0.05 m soil depth), 25% (0.15 m), and 38% (0.45 m) of the total root density in the living mulch system.

**Keywords:** maize, Italian ryegrass, living mulch, below-ground competition, roots, root density, green fluorescent protein (gfp), minirhizotrons.
Introduction

The production of maize in “living mulch systems” (Croster and Masiunas, 1998; Hooks et al., 1998) has been proposed as an environmentally sound option for suppressing weeds, minimizing soil erosion (Liebman and Dyck, 1993; Teasdale, 1996) and decreasing the extent of nitrate leaching to the groundwater (Liedgens et al., 2004a). In such systems maize may be sown into an existing grass stand; the soil between the maize rows remains covered by the grass throughout the maize growing season and even after harvest of maize if the intention is not to crop the soil immediately after.

Despite the environmental benefits, the adoption of this cropping system is limited due to reductions in the grain yield of maize (Dehaan et al., 1994; Eberlein et al., 1992; Enache and Ilnicki, 1990; Garibay et al., 1997; Liedgens et al., 2004b; Robertson et al., 1976; Zemenchik et al., 2000). On the one hand, vigorous growth of the cover crop is fundamental to cover the soil surface and suppress weeds (Dehaan et al., 1994; Enache and Ilnicki, 1990; Teasdale, 1996), but it is also considered to be the cause of the reduced grain yield of maize (Box et al., 1980; Feil et al., 1997). Consequently, different chemical and mechanical strategies for controlling the cover crop growth were studied to reduce the competition between the main and the cover crop in living mulch systems (Garibay et al., 1997; Grubinger and Minotti, 1987; Kumwenda et al., 1993; Zemenchik et al., 2000). The competition between the cover and the main crop in living mulch systems have been investigated by varying the supply of resources such as water and nitrogen (Box et al., 1980; Eberlein et al., 1992; Feil et al., 1997). These investigations have focused on shoot parameters whereas interactions between the root systems have little been studied.

In previous studies conducted in the Swiss midlands maize was sown into an Italian ryegrass living mulch, and the Italian ryegrass was controlled by mechanical (mowing) or chemical (application of herbicides) means (Feil et al., 1997; Garibay et al., 1997; Liedgens et al., 2004b). Since the shoot competition between maize and Italian ryegrass was controlled in these studies, the decrease in grain yield of maize was attributed to below-ground interactions between the two crop species (Feil et al., 1997; Garibay et al., 1997). One of the few studies in which
roots in a living mulch system have been investigated showed that the maximum root densities for monocropped maize and maize as the main crop in a living Italian ryegrass mulch were attained approximately at the anthesis of maize (Liedgens et al., 2004b). Furthermore, the root density of monocropped maize was markedly lower than that of the crop mixture (maize plus cover crop) and monocropped Italian ryegrass. Therefore, it was hypothesized that the reduced shoot growth of maize in the living mulch is related to increased allocation of carbon to root growth as a response to the lower availability of soil resources. The lack of a suitable method for determining the proportion of roots that maize and Italian ryegrass contribute to the total amount of roots of the living mulch system made it impossible to test this hypothesis.

DNA extraction (Brunner et al., 2001; Mommer et al., 2008) and tracers (Baldwin and Tinker, 1972; Jumpponen et al., 2002; Lehmann et al., 1998) have been used as methods to discriminate the roots formed by the various species in mixed plant stands. However, to better understand the effects of root interactions between maize an Italian ryegrass in a living mulch system a method is needed that allows to (i) collect a large number of samples at different soil depths throughout the growing season of the main crop, (ii) directly quantify the roots of each crop, and (iii) determine the relative arrangement of the fine roots of each crop. We have recently developed a method (Faget et al., 2009) that fulfills these requirements and, thus, makes it possible to study the spatial distribution of the roots of maize growing in an established lay of Italian ryegrass in detail.

The main goals of the present study were to determine (i) the extent to which shoot and root growth of maize are reduced by the presence of a permanent Italian ryegrass cover crop and (ii) whether the spatial distribution of the root system of maize is modified by the companion cover crop.
Material and methods

In order to observe legal restrictions for the use of genetically modified organisms in Switzerland, the experiment was conducted in a greenhouse and harvested at the onset of the anthesis of maize.

Plant material

The maize genotype ETH-M72 was genetically transformed to include the gene for gfp (ETH-M72GFP). The transformation construct contains the gfp gene flanked by the ubiquitin promoter (ubi::gfp) and the NOS terminator. It was cloned into the pUC19 vector, which contains the gene for ampicillin resistance (ampR) at the restriction sites SpeI and XbaI. The gfp gene was cloned into the cassette at the NcoI and SalI sites. The expressed GFP has been reported to have a fluorescence peak between 500 and 520 nm when excited by light at 450 to 470 nm. Information about the validation of the genetic transformation, the integration pattern of the inserted gene, and comparisons of the homozygosity of transgenic and non-transgenic controls can be found in Aulinger et al. (2003).

Italian ryegrass (Lolium multiflorum Lam. cv. Ellie) was the cover crop in the living mulch system, ETH-M72GFP was the main crop.

Experimental conditions

The experiment was conducted twice; the first run took place from 12 December 2007 to 8 April 2008 and the second one from 11 January 2008 to 16 May 2008.

The plants were grown in wooden boxes (0.60 m wide x 0.80 m long and 0.80 m high). Three minirhizotrons (60 mm outer diameter) were installed horizontally in the boxes at depths of 0.05, 0.15, and 0.45 m. They were horizontally spaced at 0.15 m from each other and from the edges of the boxes (Fig. 1).
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Figure 1: Schematic representation of the boxes and plant arrangement used in the experiment.

The boxes were filled three months before starting the experiments to enable the soil to settle, thereby avoiding effects of unsettled soil (Hutchings and John, 2004) and patches with high concentrations of nutrients (Fransen et al., 1999) on root growth. The soil was a sandy loam from a nearby field mixed with sand. According to the methods given in parentheses, the properties of the soil mixture were: 30 g kg\(^{-1}\) organic matter (Blake-Walkley), 60 g kg\(^{-1}\) clay, 110 g kg\(^{-1}\) silt, 810 g kg\(^{-1}\) sand, pH 7.7, 66.3 mg kg\(^{-1}\) assimilable K (NH\(_4\) acetate), 4.1 mg kg\(^{-1}\) P (Olsen), and 12 g kg\(^{-1}\) N (2M KCl). The mean day and night temperatures in the greenhouse were 23°C and 19°C. Plants were grown under white-light photoperiod of 16 h at 3680 lx provided by high pressure mercury lamps (Philips HPL-N-400W; Royal Philips Electronics Inc., Amsterdam, Netherlands).

Italian ryegrass was sown at a density of 6.27 g seed m\(^2\) (i.e. ~6000 plants m\(^2\)). After 40 days, one 0.30 m wide strip of Italian ryegrass in the mid-section of the boxes and perpendicular to the longer side was removed. ETH-M72\(_{GFP}\) maize was sown at a density of 10.4 seeds per m\(^2\).
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

into this strip. At the same time, ETH-M72\textsubscript{GFP} maize was sown in boxes in which the soil was left bare. The maize rows were orientated perpendicular to the minirhizotrons (Fig. 1). Hereafter, maize plants from the living mulch and from the bare soil plots will be referred to as LM and BS maize, respectively.

After sowing the maize, the remaining Italian ryegrass was cut at a height of 0.10 m at weekly intervals to exclude competition between maize and the cover crop for light. Foskal\textsuperscript{®} (P\textsubscript{2}O\textsubscript{5}, K\textsubscript{2}O, Mg, Ca, and S; Agroline, Basel, Switzerland) and ammonium nitrate (NH\textsubscript{4}NO\textsubscript{3}) were evenly applied to the soil surface before sowing the maize supplying 1.4 g P, 8 g K, 0.57 g Mg, 0.20 g Ca, 0.87 g S, and 1.1 g N per m\textsuperscript{2}. The water content of the soil was estimated by time domain reflectometry (EC-5 Soil Moisture Sensor, Decagon Devices Inc., Pullman, WA, USA) and maintained close to field capacity by irrigation.

\textit{Sampling and screening of data}

The shoots of maize were cut approximately 83 days after sowing at ground level when the experiments were terminated. The maize and Italian ryegrass shoots were dried at 60\textdegree C until constant weight. The biomass yield reported for Italian ryegrass represents the sum of the biomass yields of the weekly cuttings and includes the biomass at the end of the experiments. The leaf area of maize was measured at the termination of each experimental run with a leaf-area meter (LI-COR 3100, Lincoln, NE, USA). The leaf area of Italian ryegrass was not determined.

Root images were recorded at the minirhizotron – soil interface using two camera configurations for distinguishing roots of maize and Italian ryegrass. One camera configuration allowed for the sampling of images that display all structures at the minirhizotron-soil interface while the second configuration allowed for identifying precisely the roots of ETH-M72\textsubscript{GFP} as a result of the GFP expression (Faget et al., 2009). The use of identical handles for both camera configurations enabled us to repeatedly sample images of both types at the same position in the minirhizotrons over the entire duration of the experiment. The images were obtained from the upper surfaces of the minirhizotrons. At each of the soil depths analyzed, the first image (19.5 mm wide) of the sequence was taken immediately under the maize row. From there, the camera covered a strip of 312 mm towards the border of the plot; images were taken at 12 positions. The
first six positions were below the strip on which the Italian ryegrass cover was removed shortly before maize sowing (maize zone, hereafter). The remaining six images were taken below the living ryegrass mulch (grass zone, hereafter).

The number of roots (i.e. number of root incidences on the minirhizotron interface) for each of the sampled images was determined according to Upchurch and Ritchie (1983). Although this estimate can be obtained from single minirhizotron images, the accuracy of identifying root structures in images increases when images acquired at the same position are captured across a set of sampling dates (Smit et al., 2000). Therefore, all images recorded at a single minirhizotron position over the entire growing season were organized into an image time series (ITS). Images obtained with each camera configuration were intercalated in each ITS. Since no objective visual criteria exist to determine whether a root is functional (Smit et al., 2000), all roots identified in an image were accounted for in each ITS. Thus, the obtained response variable was the cumulative number of roots observed per image since the sowing date. This was converted into a surface unit (cm\(^2\)) and is referred to hereafter as root density (roots cm\(^{-2}\)).

**Data analysis**

Root density was analyzed throughout the growing season of the crops and for the class distances from the maize row. An estimate of the root density for the soil profile is provided in Fig. 2; this was obtained as the mean root density for the three studied soil depths. In addition, we calculated ratios of root density in the grass zone to root density in the maize zone.

For each experimental run (n=2) we used a completely randomized block design with five replications and two treatments. The treatments were maize grown as a monocrop on bare soil (BS maize) and maize sown into living ryegrass mulch (LM maize). The analysis of variance of all the datasets was performed with R (R Development Core Team, 2007). Bars presented in graphs are standard errors of the mean.
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Results

Effects of Italian ryegrass on the shoot and root growth at the anthesis of maize

Italian ryegrass as living mulch significantly reduced the biomass yield, leaf area, and root density of maize (Fig. 2).

Values are mean ±SE of 10 measurements

**Figure 2:** Means of shoot and root parameters of maize and Italian ryegrass measured at harvest in two experimental runs. Root density is the mean of three soil depths (0.05, 0.15, and 0.45 m).

The biomass yield of BS maize was, with 354 g m⁻², the higher than that of Italian ryegrass (156 g m⁻²) and LM maize (155 g m⁻²). The leaf area, which was measured only for maize, was higher for BS maize (0.96 m² m⁻² soil) than for LM maize (0.58 m² m⁻² soil). In contrast to the biomass yield, the highest root density was found for Italian ryegrass (1.43 roots cm⁻²), followed by BS maize (1.13 roots cm⁻²) and LM maize (0.66 roots cm⁻²). In interpreting these data, it must be borne in mind that the final root density of the grass in the living mulch
system was the result of root growth before and after the sowing of maize. The biomass yield, leaf area and root density of LM maize were 56%, 39%, and 41%, respectively, lower than that of BS maize. Therefore, the relative reduction that was found for LM maize as compared to BS maize was almost identical for the assimilatory organs (i.e. leaf area and root density).

**Effects of Italian ryegrass on the spatial distribution of maize roots at anthesis**

Figure 3 shows the mean root density of LM maize, BS maize and Italian ryegrass at three different soil depths, namely 0.05, 0.15, and 0.45 m. Root density of BS and LM maize was significantly different at all depths; the root density of LM maize was 36%, 46% and 42%, respectively, lower at 0.05, 0.15 and 0.45 m compared to BS maize. Furthermore, maize roots accounted for 32% (0.05 m), 25% (0.15 m), and 38% (0.45 m) of the total root density in the living mulch plots.

The maximum root density of Italian ryegrass and maize was observed at 0.05 m; for maize this was independent of the cropping method. In all crops, root densities at 0.15 and 0.45 m depth were similar. Except for 0.45 m depth, values were higher for Italian ryegrass than for BS maize in the two upper soil layers, but the root density of Italian ryegrass was always significantly higher than that of LM maize. This suggests a strong negative effect of Italian ryegrass on the below-ground growth of LM maize.

Immediately below the soil surface, at 0.05 m depth, the root density of maize decreased with increasing distance from the maize row, irrespective of the maize cropping system (Figure 4).
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Values are mean ±SE of 120 measurements.

**Figure 3:** Means of root density by soil depth of maize in bare soil (BS) and maize and Italian ryegrass in a living mulch system (LM) measured at the harvest of two experimental runs.
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Values are mean +SE (LM Maize) or -SE (BS Maize) of 10 measurements.

**Figure 4:** Means of root density for row-distance classes of maize grown in bare soil (BS) and in a living mulch system (LM) at the harvest of two experimental runs. Shadow areas indicate row-distance positions below the soil surface cultivated with ryegrass.
Root densities of LM maize were consistently lower than those of BS maize, but the differences were only small at distances of more than 200 mm. At soil depths of 0.15 m and 0.45 m, the effect of distance on root density from the row depended on the maize cropping system. The differences between the maize cropping systems were relatively small at low distances from the maize row. At distances of more than 150 mm, however, BS maize clearly formed more roots than LM maize. In contrast to the 0.05 m soil depth, root density at 0.45 m soil depth increased from about 150 mm distance from the maize row for BS maize.

We calculated the ratios of root density in the grass zone to root density in the maize zone. BS maize had ratios of 0.57, 1.80, and 1.88 at depths of 0.05, 0.15, and 0.45 m, respectively. The respective values for LM maize were 0.40, 0.38, and 1.80. The differences between BS maize and LM maize were non-significant at p=0.05. These figures indicate that, immediately below the soil surface, relatively more maize roots occurred in the maize zone, irrespective of maize cropping system. In contrast, relatively more maize roots were formed in the grass zone in deep soil layers in both maize production systems. At 0.15 m depth, however, there were marked differences between the maize cropping systems.

Dynamics of the root growth of maize in bare soil and in the living mulch

The temporal patterns of root density below the maize and grass zones are shown in Figure 5. The root density of BS maize increased at the three soil depths until anthesis. The root density of LM maize was generally lower than that of BS maize throughout the growing season. In the maize zone, the lag in root growth of LM maize compared to BS maize markedly decreased with increasing soil depth. (Figs. 5a-c). This effect was not observed in the grass zone (Figs. 5d-f).
Chapter 3: Root growth of maize in a living Italian ryegrass mulch

Figure 5: Means of root density at depths of 0.05 (a, d), 0.15 (b, e) and 0.45 m (c, f) of maize in bare soil (BS) and in a living mulch system (LM) of two experimental runs. Root density was obtained for row-distance positions. Values are mean ± SE (LM Maize) or -SE (BS Maize) of 60 measurements.
Discussion

The reduction in the shoot biomass yield of maize (Fig. 2) agrees well with results found in other studies in which maize was grown in living grass mulches (Feil et al. 1997; Garibay et al. 1997; Liedgens et al. 2004b). Reduced above-ground biomass of LM maize, compared to BS maize, was due to a smaller photosynthetic area, which can be inferred from the smaller leaf area of LM maize (Fig. 2). Similar reductions in leaf area were found by Liedgens et al. (2004b) in a three-year outdoor lysimeter study in which LM maize showed a faster leaf senescence than BS maize after anthesis. Unfortunately, we could not investigate the postfloral period due to legal restrictions associated with the use of transgenic maize.

In this study, it was significant that the living grass mulch had approximately the same adverse effects on leaf area and total root density (Fig. 2). Root growth of maize in living mulch systems has been investigated in previous studies, but the maize root density was determined by calculating the difference between total root density of the living mulch system and monocropped Italian ryegrass (Liedgens et al. 2004b). This approach represents only a rough estimate of the contribution that maize makes to the total root density in the living mulch system. By discriminating between GFP-derived fluorescent and non-fluorescent roots (Faget et al. 2009), we were able to directly quantify the relative proportion of maize and Italian ryegrass roots in the living mulch in situ.

The spatial distribution of the roots of crops in mixtures has been analyzed in a few studies using methods that rely on the separation of rooting volumes by barriers (e.g., Li et al. 2007; Thorsted et al. 2006) or trenches (e.g., Li et al. 2006; Ludwig et al. 2004). Such methods dramatically disturb the soil environment and can lead to artefacts in plant growth (Connell 1990; McPhee and Aarssen 2001). Our results were obtained with minirhizotrons, a method that minimizes the interference to root growth (Taylor et al. 1990). Thus, our study provides reliable information about the spatial distribution of maize roots in a crop mixture grown on undisturbed soils, which may further understanding and management of the coexistence of plants in crop mixtures (Schenk 2006).
It may be assumed that the roots of LM maize preferentially grow in the soil below a strip where Italian ryegrass has been removed (we have previously defined this area as the maize zone). An indicator of a shift of LM maize root growth to the maize zone would be a variation in the ratio of root density in the grass zone to root density in the maize zone, between LM maize and BS maize. We found differences in the relative spatial distribution, especially at a soil depth of 0.15 m, but the differences were not significant. Consequently, further research, ideally in an outdoor environment with 10 observation depths at minimum, is needed to elucidate the nature of this effect. In interpreting the results for root distribution, it should be noted that the fertilizers were evenly distributed on the soil surface in the present study. In farmers’ fields, however, nitrogen fertilizers could be broadcasted or applied to the maize rows (Malhi et al. 2001). The latter method is likely to increase the production of roots below the maize rows (Chassot et al. 2001).

Differences in root density between BS maize and LM maize occurred as early as at the seedling stage (Fig. 5). This effect was likely caused by nitrogen deficiency due to nitrogen removal by grass from the living mulch system before the maize was planted.

Root density decreases with soil depth, but it may be higher at a depth of 0.15 m than at 0.05 m for monocropped maize (Liedgens and Richner 2001) or for maize and Italian ryegrass in a maize - Italian ryegrass living mulch system (Liedgens et al. 2004b). Such differences may be explained by the fact that the soil surface is usually dryer in outdoor experiments that have erratic rainfall (Liedgens and Richner 2001; Liedgens et al. 2004b) than in greenhouse studies, where the soil surface is frequently watered. The root densities in our study were slightly lower than those reported by other experimenters (e.g., Liedgens and Richner 2001; Liedgens et al. 2004b; Liedgens et al. 2000; Pages and Pellerin 1994; Smit and Groenwold 2005). For example, for the BS maize, we found 1.7, 0.8, and 0.9 roots cm\(^{-2}\) at soil depths of 0.05 m, 0.15 m, and 0.45 m, respectively. By contrast, Smit and Groenwold (2005) found densities of 2.3±1.5, 2.3±2.1, and 0.6±0.4 roots cm\(^{-2}\) at the same respective soil depths. Continuously decreasing root densities of maize in the topsoil and a pronounced response to increasing soil depth in the subsoil were similarly found in other studies using minirhizotrons (Liedgens and Richner 2001; Liedgens et al. 2004b).
Conclusions

The present study demonstrates that the use of transgenic maize expressing GFP combined with the minirhizotron technique allows for reliably tracing the dynamics of maize root growth planted into established Italian ryegrass sods. A potential drawback of the GFP method is that, in many countries, it can only be applied in confined environments due to legal restrictions.

This study provides unique data on the growth of maize roots into an established Italian ryegrass sod and it demonstrates that the leaf area and mean root density of maize cropped in a living mulch system were almost identically reduced by the companion crop.
General Conclusion and Outlook

General Conclusion

Roots are essential for plant growth due to their role of anchorage and absorption of water and nutrients. This hidden part of plants is less studied than the corresponding shoot due to its relative inaccessibility in the soil without perturbing the environment. Plant species differ in the temporal and spatial exploitation of the soil, interacting in crop stands as well as in natural systems with other plants. However, there is no method to follow in a regular manner the belowground interactions in between different plants or species in-situ. The main questions were “How to distinguish roots belonging to different plants?”,” “How do roots interact?” .Here we demonstrated that using transgenic plants expressing a fluorescent protein; combined with the minirhizotron technique brings a first reliable answer to those questions.

By using plants transformed with the GFP and constructing a specific minirhizotron camera, albeit is possible from now on to distinguish roots from different plants in the soil. The prerequisite for the application was the use of the green fluorescent protein that has not been applied before for agronomic and ecological root studies.

The two cameras developed are of a similar construction but with different goals. The conventional minirhizotron camera type was already available in the market; however the one constructed here is easily affordable in terms of price and highly flexible for further potential modifications. It allows taking digital images of the soil root interface with a white light as used in the conventional minirhizotron systems. The GFP minirhizotron camera system is a new powerful tool which allows unequivocally identifying roots from plants expressing the GFP. Therefore, we demonstrated that it is possible to develop an affordable imaging system for capturing imaging of conventional and fluorescent roots growing in the soil.

The conventional images were of similar quality as those from commercial equipment and fulfill the requirements of software to analyze root images. The small size of the captor (640 *
General Conclusion and Outlook

480 pixels) was not a limitation for the latter. The roots, soil, and even soil interstices were highly visible and images were similar than the ones obtained with commercial devices. The quality of images obtained with the GFP device was the same standard as those obtained with the conventional device. Roots from transformed plants were exclusively visible in a bright green color. It was a strong additional advantage that with this light the background is black; thus a better contrast exists that increases the precision to distinguish the roots from the background compared to the conventional images. Another advantage concerns the fine roots; because of their transparency, they are not completely visible in conventional images and parameters such as root density are often underestimated. In images obtained with the GFP minirhizotron camera, these fine roots are also visible in a bright green. Therefore, this method represents an improvement for the minirhizotron studies by increasing its accuracy. Interestingly, it was furthermore possible to identify precisely the meristematic tissues, i.e. root tips and lateral branch insertion because there the fluorescence is stronger than in the others parts of the roots.

By growing the genetically transformed maize genotype ETH-M72 GFP with its corresponding wild type, Italian ryegrass or soybean, we demonstrated that this method allows indeed an accurate non destructive observation of different root systems. The green emitted light was only observable in the transformed plants and enabled to investigate the dynamics of the relative distribution of roots between intraspecific or interspecific neighbors. Using root fluorescence of genetically transformed plants with minirhizotrons seems to be, compared to other methods the simplest one to study the relative distribution of roots in mixed plant stands. In addition to distinguishing roots of different plants, the root density quantified in images using the GFP system was 28% higher than that obtained with the conventional method. Therefore it can be envisioned that GFP transformed plants may be even useful in single plant studies when morphological developments have to be screened with a high descriptive accuracy.

The main study with this new method was conducted for maize cropped in a living mulch of Italian ryegrass. This is a promising system for the sustainable production of maize. Maize expressing GFP was grown either alone in bare soil or together with Italian ryegrass in boxes. By using the GFP minirhizotron method, it was possible to measure the root proportion of each
species in space and time until anthesis. As known before, Italian ryegrass decreased strongly the root density and leaf area of maize by 41% and 39%, respectively. This is unique information that answers a long open question on how the roots of a staple crop establish in the soil occupied by living grass; it could have never been answered without using our novel approach that allows quantitatively discriminating roots and studying their relative distribution. It was another advantage that the dynamics of this competitive distribution could be followed. These first results already showed that Italian ryegrass induced a delay in the root growth of the main crop; the temporal distribution was more affected than the spatial distribution. This paves the way to devise strategies to increase the sustainability of multi-species cropping systems in the future.

**Outlook**

It will be important to conduct similar experimentation with different plant species as a cover crop, for example legumes where the association could be more beneficial for the main crop by less competition for N and even a possible nutrient transfer.

At the present state, more fluorescent proteins with different wave lengths, i.e. colors exist. Thus this novel approach to identify and study roots could be extend to a larger plant communities with more than two species together when many plants with different fluorescent proteins as blue, cyan, yellow, red can be used with an easy adaptation of minirhizotron camera, tracking all the different roots system to study the belowground interactions in a whole community.

We noticed in some pictures that the fluorescence of meristematic tissues, i.e. root tips and lateral branch insertion was stronger than in the others parts, and in opposite older roots were loosing gradually their fluorescence. This characteristic could be used to adapt this new method to study the senescence of root systems. This will depend on finding a relationship between root activity and fluorescence intensity. Most of the common approaches with minirhizotron studies imply that a huge quantity of images has to be analyzed. Due to the heterogeneous soil background and the poor visibility of some fine roots, a manual drawing of root has to be done. With the GFP minirhizotron technique, a more accurate
identification of roots is possible due to the better contrast between the roots and the background. The clear contrast between the black background and the fluorescent green roots could allow for applying a simple image analysis procedure to track the roots automatically. This could bring an advance in this domain where a lot of time is needed to analyze every image of an experiment.

By the possibility to study root interaction in space and time and extending this method to new fields of research, this novel approach literally brings light into this underground environment.
References


References


References


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