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David Morais

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THÈSE

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Les déterminants des phases épidémiques précoces de la septoriose du blé (*Zymoseptoria tritici*) : quantité, efficacité et origine de l'inoculum primaire

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Présentation générale

Présentation générale

L'unité BIOGER (Biologie et Gestion des Risques en agriculture - Champignons Pathogènes des Plantes) développe des programmes de recherche sur les champignons parasites des plantes à des échelles allant du gène au paysage. L'enjeu de ces recherches est de concevoir des méthodes de gestion qui prennent en compte aussi bien les mécanismes des interactions plante-pathogène que leur évolution dans différents systèmes de culture.

L'équipe "Epidémiologie des maladies fongiques du blé" s'intéresse aux principales maladies foliaires de cette culture : la rouille brune (*Puccinia triticina*), la rouille jaune (*Puccinia striiformis*) et la septoriose (*Zymoseptoria tritici*). Au travers de ces trois modèles biologiques, elle aborde de manière pluridisciplinaire (mycologie, agronomie, génétique, écologie, biophysique et modélisation) les questions suivantes : Comment se développent les maladies à différentes échelles de temps et d'espace ? Quels facteurs biotiques et abiotiques influencent leur dynamique ? Quels principes et moyens de gestion peuvent être mis en place pour limiter leurs conséquences ?

L'équipe consacre une partie importante de ses programmes à étudier le commencement et la récurrence des épidémies de septoriose et de rouille brune du blé, dont les agents possèdent des caractéristiques épidémiologiques contrastées. Par exemple, entre deux épidémies annuelles, le parasite hémibiotrophe *Z. tritici* survit sur les résidus de blé et le parasite biotrophe *P. triticina* sur des repousses de blé. L'objectif est de comprendre le fonctionnement pluriannuel de ces épidémies, en identifiant la nature et l'origine de l'inoculum, en évaluant la capacité de survie de l'agent pathogène, et en caractérisant les mécanismes impliqués dans les contaminations les plus précoces. L'enjeu est à la fois scientifique (résolution d'une "boîte noire" épidémiologique) et pratique (modèles de prévision, modèles de gestion).

La plateforme "BiogeR-Syst" est intégrée au réseau INRA (R-Syst) chargé d'implémenter et maintenir une base de données de systématique. Elle développe des approches d'identification moléculaire de différents champignons phytopathogènes à partir d'échantillons issus de diverses matrices (tissus vivants, résidus, sol, aérosols, etc.).

De nouvelles problématiques de biosécurité ont émergé en santé végétale, conduisant à des besoins d'expertise sur les risques épidémiques auxquels sont exposés les agro-

écosystèmes, qu'ils résultent de menaces naturelles (épidémies émergentes ; Desprez-Loustau et al., 2007), accidentelles (justifiant les mesures de quarantaine végétale ; Schrader & Unger, 2003), ou intentionnelles (actes de malveillance ou d'agroterrorisme ; Suffert, 2002 ; Madden & Wheelis, 2003 ; Waage & Mumford, 2007). L'équipe Epidémiologie s'est positionnée sur cette thématique il y a quelques années, en ayant pour objectif la caractérisation du risque agroterroriste : typologie des menaces, hiérarchisation de cibles, développement de modèles d'analyse de risque (Latxague et al., 2007 ; Suffert et al., 2008). La relation entre l'évaluation de ce type de risque (Suffert et al., 2009 ; Boumrar, 2013) et les travaux de recherche ayant pour finalité la conception de méthodes de gestion de maladies courantes, est loin d'être évidente. C'est pourtant au sein d'un seul et même projet financé par l'Union européenne (PLANTFOODSEC ; Gullino et al., 2011 ; Reynaud et al., 2012) que les deux problématiques se sont rejointes. Serait-on capable de différencier l'introduction naturelle ou accidentelle d'un agent pathogène d'une introduction intentionnelle ? La réponse est probablement "non", puisqu'à la question "Comment commence une épidémie naturelle ?" il ne peut avoir de réponse unique et définitive. Théoriquement, une seule contamination est suffisante pour déclencher une épidémie. En pratique, les choses sont plus complexes puisque les connaissances sur les mécanismes et les facteurs impliqués dans le déclenchement (ou non) d'une épidémie sont encore insuffisantes. Quel type et quelle quantité d'inoculum sont nécessaires ? D'où vient-il ? Comment est-il dispersé ? Comment entre-t-il en contact avec les plantes hôtes ? Que se passe-t-il entre les premières contaminations et la phase de développement épidémique de la maladie ? Combiner différentes approches expérimentales pour tenter de répondre à ces questions permettrait d'accroître la capacité de réaction des acteurs concernés, dans une perspective ordinaire (diminuer l'impact d'épidémies récurrentes sur les productions végétales) mais aussi moins conventionnelle (identifier les failles de filières agricoles exposées à des risques épidémiques émergents).

La biologie légale (en anglais *bioforensic*) appliquée aux agents phytopathogènes prend une importance croissante dans le contexte de la biosécurité des cultures, notamment aux États-Unis (Fletcher et al., 2006). Cette discipline consiste à s'appuyer sur des méthodes scientifiques pour mener des investigations visant à identifier l'origine d'un foyer de maladie et, le cas échéant, confondre les auteurs d'actes délictuels ou malveillants (Rasko et al., 2011). La démarche se concentre sur l'identification de l'origine de souches pathogènes par des méthodes moléculaires (par exemple analyse de distances phylogénétiques), postulant que toute interprétation épidémiologique n'est possible que si l'on dispose d'un marquage précis

et discriminant des souches. Si la démarche peut conduire à émettre des hypothèses probantes sur l'émergence d'épidémies humaines et de zoonoses et sur leur divergence en fonction du temps (Chan, 2002 ; Faria et al., 2014), elle s'avère souvent moins concluante en santé végétale. Les souchothèques sont limitées. Les outils de diagnostic moléculaire et les marqueurs ne sont pas toujours disponibles ou adaptés. Et, surtout, les moyens alloués à ces recherches sont corrélés aux enjeux, qui sont à l'évidence plus forts en santé humaine et animale.

"Tracer" un agent phytopathogène en conditions naturelles ou "remonter sa piste" constitue un défi technologique. Il est généralement nécessaire de s'appuyer sur des méthodes indirectes, d'inférence statistique ou expérimentale. Le point fort de l'épidémiologie végétale est certainement que, pour comprendre comment commence une épidémie, il est possible d'expérimenter "au champ" sans risque. Les maladies les plus courantes permettent cela, à l'inverse des maladies exotiques (dites de quarantaine) ou des agents de la menace agroterroriste dont l'étude en conditions naturelles est exclue. Pour ces raisons, la septoriose et la rouille brune du blé ont été retenues dans PLANTFOODSEC comme modèles d'étude génériques. Ma thèse, consacrée à l'étude des déterminants des phases précoces des épidémies de septoriose, a été réalisée à BIOGER dans le cadre de ce projet. J'ai travaillé sous la responsabilité directe de Frédéric Suffert (responsable de l'équipe "Epidémiologie des maladies fongiques du blé") et de Valérie Laval (responsable de la plateforme BiogeR-Syst), en collaboration avec Anne-Sophie Walker (scientifique dans l'équipe "Antifongiques, mode d'action et résistance"), et sous la direction d'Ivan Sache (Professeur de Pathologie Végétale et Épidémiologie à AgroParisTech).

Chapitre I

Contexte, enjeux et objectifs de la thèse

Chapitre I - Contexte, enjeux et objectifs de la thèse

1. Pourquoi s'intéresser à l'inoculum primaire en épidémiologie végétale ?

1.1. Quelles sont les principales caractéristiques d'une épidémie ?

L'épidémiologie végétale est la discipline qui s'intéresse à la dynamique des maladies des plantes dans le temps et dans l'espace (Zadoks & Schein, 1979 ; Jones, 1998) et les facteurs biotiques et abiotiques qui les influencent. Une maladie résulte de l'interaction entre une population pathogène, un peuplement végétal et des conditions environnementales (triangle épidémique). Les épidémies doivent être analysées dans un environnement fortement influencé par les activités humaines, en particulier par les pratiques culturales, qui constitue le quatrième sommet du tétraèdre épidémique (Figure 1).

Dans le cas de maladies causées par des champignons, les facteurs environnementaux ayant la plus grande influence sont la température et l'humidité, mais on peut aussi citer le vent (qui permet la dispersion des spores et peut modifier localement les conditions de température et d'humidité), les radiations solaires (qui peuvent inhiber ou stimuler la germination de certaines spores) et les caractéristiques physico-chimiques du sol (pour ce qui concerne les maladies telluriques). Le développement d'une maladie est affecté par la composante "humaine", directement ou indirectement, par exemple par les choix variétaux (pour leur potentiel de rendement ou leur niveau de résistance aux maladies), par la densité de peuplement ou la modulation des dates de semis (Agrios, 2005). Les pratiques culturales ainsi que l'utilisation de substances chimiques ou d'agents de lutte biologique conditionnent également le développement des populations pathogènes.

Vanderplank (1963), un des fondateurs de l'épidémiologie végétale moderne, a proposé dans son ouvrage de référence "*Plant diseases: epidemics and control*" une première vision de l'étude d'une épidémie végétale en considérant la dimension spatiale et la dimension temporelle indépendamment. Zadoks (1979, 2001) a rassemblé ses deux dimensions (ordres 0, 1 et 2 d'une épidémie, qui correspondent à différentes échelles) à la suite de ses travaux sur les dynamiques focales de maladies à des échelles micro, méso et continentales (Figure 2).

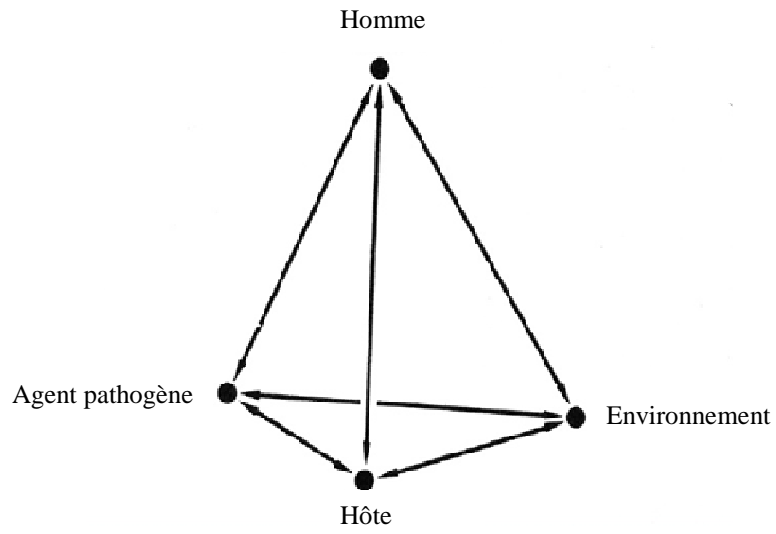


Figure 1 - Le tétraèdre épidémique (d'après Zadoks & Schein, 1979).

Il est important de déterminer la nature d'une épidémie, monocyclique ou polycyclique, pour comprendre le développement d'une maladie. Les épidémies monocycliques résultent de l'action d'un seul pool d'inoculum au début, qui diminue ensuite au cours d'une saison culturale (pas de renouvellement) ; les épidémies polycycliques démarrent par l'action d'un pool d'inoculum et se poursuivent ensuite par plusieurs cycles de multiplication emboîtés (Bousset & Chèvre, 2012). Un autre facteur déterminant la dynamique temporelle d'une épidémie est la capacité de survie de l'agent pathogène entre deux épidémies. Où se trouve-t-il et quelles conditions environnementales influencent sa survie ? Pour certains pathosystèmes, l'existence d'hôtes secondaires ou alternants, sur lesquels se déroule par exemple la reproduction sexuée, peut contribuer à augmenter la durée d'une épidémie au-delà de la saison végétative de l'hôte principal. Certains hôtes alternants ont été identifiés plus ou moins récemment, comme l'épine-vinette (*Berberis sp.*) pour la rouille jaune à *Puccinia striiformis* (Jin et al., 2010) et la rouille noire à *Puccinia graminis* (De Bary, 1865 ; Roelfs, 1982), le pigamon (*Thalictrum sp.*) pour la rouille brune à *Puccinia triticina* (D'Oliveira, 1940 ; D'Oliveira & Samborski, 1966) ou le mélèze (*Larix sp.*) pour la rouille du peuplier à *Melampsora larici-populina* (Klebahn, 1902).

Les composantes spatiales d'une épidémie se concentrent généralement sur la capacité de dispersion de l'agent pathogène (Fitt et al., 1989) et ses conditions de survie, qui déterminent l'échelle de l'unité expérimentale à considérer pour l'étude de la maladie. Les échelles spatiales nécessaires à l'étude d'une épidémie ou d'une population pathogène peuvent aller de l'échelle foliaire à l'échelle continentale (Linde et al., 2002 ; Brown & Hovmøller, 2002).

Ce qui précède conduit à s'interroger sur la durée d'une épidémie (une année culturale ou plusieurs années consécutives) et de son étendue dans l'espace (parcelle, bassin versant, région, continent), et, de façon plus générale, sur les échelles de temps et d'espace à prendre en compte pour étudier ses phases précoces : le début de la saison culturale, l'intersaison et/ou la fin de la saison précédente ? Une parcelle locale ou un ensemble de parcelles éloignées les unes des autres ? Les réponses à ces questions dépendent des caractéristiques biologiques de l'agent pathogène considéré, et notamment des propriétés de l'inoculum qui est responsable du commencement d'une épidémie : l'inoculum primaire. Théoriquement, une seule contamination est suffisante pour déclencher une épidémie. En pratique, le mécanisme est plus complexe et les connaissances sur les mécanismes et les facteurs impliqués dans le déclenchement (ou non) d'une épidémie sont encore insuffisantes.

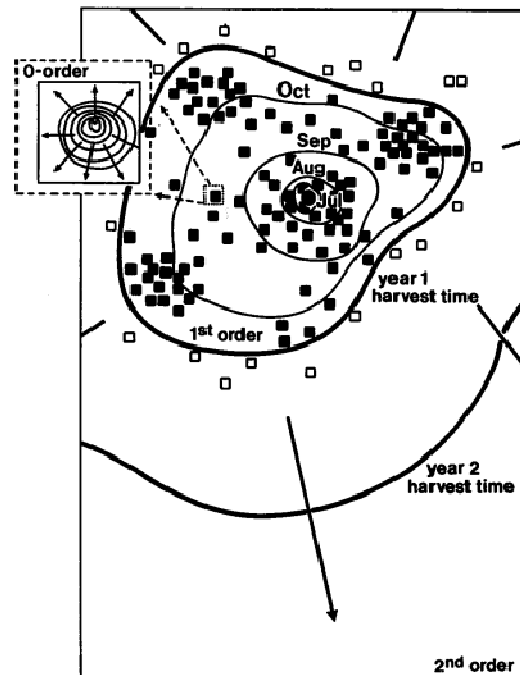


Figure 2 - Schéma de trois ordres de foyers épidémiques. Au centre, l'épidémie d'ordre 0 (niveau micro) ou le foyer s'élargit et se développe sur une saison et dont les lignes successives de la même sévérité (isopathes) sont dessinées. La taille de ce foyer est de un à quelques mètres de diamètre. Les carrés noirs au sein de la ligne large intérieure montrent les résultats de l'épidémie de premier ordre, se développant sur une saison (niveau méso), avec deux isopathes minces. Les carrés blancs entre les deux dernières lignes larges représentent les premiers résultats de l'épidémie de second ordre (niveau continental), qui se développe au cours des années successives. La taille de ce foyer peut s'étendre jusqu'à tout un continent (d'après Zadoks, 2001).

Comment entre-t-il en contact avec les plantes hôtes ? Que se passe-t-il entre les premières contaminations et la phase de développement épidémique de la maladie ? Autant de questions auxquelles les épidémiologistes cherchent à apporter des réponses.

1.2. Comment débute une épidémie ?

La question "Comment débute une épidémie ?" est très large et n'a pas de réponse unique. Pour répondre à cette question, il est d'abord nécessaire de définir ce qu'est "le début" d'une épidémie. Cela peut sembler trivial lorsque l'on s'intéresse à des épidémies annuelles (7 à 10 mois de culture) causées par des agents pathogènes qui n'ont qu'un seul hôte durant leur cycle de vie. Dans ce cas, le bon sens pousse à considérer qu'une épidémie débute dès la première infection de tissus hôtes (par exemple à la levée du blé), se traduisant quelques jours ou semaines après par l'apparition des premiers symptômes. La fin d'une épidémie annuelle peut coïncider avec la disparition des plantes hôtes, par leur destruction par la maladie ou plus simplement par leur récolte (la moisson dans le cas du blé).

Il est possible d'aller encore plus loin dans l'analyse de ce que représente le "début d'épidémie" en essayant de le définir par rapport aux autres phases épidémiques, et notamment "la fin". Le début et la fin d'une épidémie sont définis par ce qu'il a avant et après : l'absence de maladie. En revanche, aucun critère simple ne permet de définir le moment où s'achève le début d'une épidémie, par exemple lorsque le développement maladie devient exponentiel. Dans le cas d'une maladie polycyclique, le moment où la quantité d'inoculum primaire qui a provoqué les premières infections devient inférieure à la quantité d'inoculum secondaire peut être retenu. Une dimension spatiale peut même être ajoutée à ce critère, comme le moment où la quantité d'inoculum primaire produit localement devient inférieure à la quantité d'inoculum secondaire provenant de l'extérieur. Au final, il est nécessaire de tenir compte de la nature des différents types d'inoculum et de leur capacité de dispersion respective. Dans le cas de la septoriose du blé, la fin du début d'une épidémie a ainsi été définie comme étant le moment où la quantité d'inoculum secondaire (transporté à courte distance) produit sur les plantes hôtes d'une parcelle en monoculture (précédent blé) dépasse la quantité d'inoculum produit sur les résidus de la culture précédente (Suffert & Sache, 2011).

1.3. La notion d'inoculum primaire

En épidémiologie végétale, l'inoculum primaire (en anglais *primary inoculum*) est un élément clé puisqu'il est, par définition, responsable du commencement des épidémies. L'inoculum peut être défini comme la ou les formes de l'agent pathogène qui arrivent au contact de leur hôte végétal et le contamine (Agrios, 2005). Chez les champignons, l'inoculum fait référence à une propagule (une spore ou un sclérote multicellulaire) et, de façon plus générale, à un génotype unique (un seul individu ou plusieurs clones issus d'un cycle de reproduction asexuée) ou à un ensemble de génotypes (par exemple issus d'un cycle de reproduction sexuée).

Les différentes dénominations utilisées dans la littérature pour qualifier l'inoculum primaire, plus ou moins explicitement, se réfèrent à sa nature (*ascospores* (Shaw & Royle, 1989 ; Trapero-Casas et al., 1996 ; Gutierrez & Shew, 1998), *conidia* (Carisse et al., 2009, 2011 ; Luchi et al., 2013), *basidiospores* (Bartz et al., 2010 ; Kaneko et al., 2014)), ou à son mode de dissémination (*airborne inoculum* (Shaw & Royle, 1989 ; Calderon et al., 2002 ; Rogers et al., 2009 ; Karolewski et al., 2012 ; Duvivier et al., 2013), *seedborne inoculum* (Bennett et al., 2005, 2007 ; Fontaine et al., 2010) ou *soilborne inoculum* (Suffert & Montfort, 2008 ; Haegi et al., 2013)). Un nom générique comme *initial inoculum* (Beest et al., 2008 ; Zheng et al., 2013), *starting inoculum* (Gobbin et al., 2003) ou *propagule* (Kaczmarek et al., 2012) peut aussi être employé.

L'inoculum primaire est souvent considéré par les épidémiologistes comme une boîte noire. En effet, malgré son impact avéré sur la précocité, la sévérité et le développement des épidémies, il est difficile à identifier et à caractériser. Il peut être défini par son implication dans certains des processus qui conditionnent le développement d'une épidémie : "les propagules ou les structures végétatives d'un agent pathogène causant des foyers primaires de maladie plutôt que des foyers secondaires" (Shurtleff & Averre, 1997), ou comme étant "les formes pathogènes qui survivent à des conditions extrêmes (hiver ou été) ou ses spores causant la première infection à l'automne ou au printemps" (Agrios, 2005). De sa quantité initiale et de son état de conservation après survie en absence d'hôte dépend, dans une certaine mesure, l'intensité de la maladie. On considère ainsi que plus les propagules infectieuses seront nombreuses, plus l'épidémie sera précoce (Jordan & Allen, 1984 ; Suffert & Sache, 2011) et sévère (Johnson, 1990 ; McMullen *et al*, 2008).

La nature exacte de l'inoculum primaire, son efficacité vis-à-vis de la plante hôte et sa source (ou origine) ont fait l'objet de nombreuses études (Bretag et al., 2006; Zhan et al., 2008; Suffert *et al.*, 2011; Chen et al., 2014).

1.4. Inoculum primaire et échelles de temps : infections primaires vs secondaires, épidémies monocycliques vs polycycliques, récurrence pluriannuelle des épidémies

Dans le cas d'une maladie monocyclique, l'agent pathogène ne se reproduit qu'une seule fois au cours d'une épidémie à partir de l'inoculum primaire généralement issu de la culture précédente (pas ou peu d'infections secondaires). C'est le cas de parasites de cultures annuelles importantes comme le colza (phoma causé par *Leptosphaeria maculans* et pourriture blanche causée par *Sclerotinia sclerotiorum*) ou le blé (fusariose de l'épi causée par un complexe *Fusarium* sp. / *Microdochium* sp.), dont l'inoculum primaire est dispersé par voie aérienne. Dans le cas d'une maladie polycyclique, l'agent pathogène réalise plusieurs cycles de reproduction par saison après les premières infections par l'inoculum primaire. La quantité d'inoculum secondaire augmente après chaque cycle infectieux (Agrios, 2005). Chez la majorité des maladies polycycliques (par exemple pour les rouilles, oïdium, septoriose et helminthosporiose du blé), l'inoculum primaire est constitué de spores (souvent sexuées) transportées par le vent. Pour certaines maladies, il est important de considérer le développement d'une maladie sur plusieurs saisons végétatives, en particulier dans le cas de monocultures de plantes annuelles (pas de rotation) ou de cultures pérennes. L'inoculum produit au cours d'une saison peut en effet être actif au cours de la suivante, conduisant parfois à un accroissement de sa quantité au fil des années. Les épidémies qui découlent de ces conditions sont qualifiées d'épidémies polyétiques (Pfender & Alderman, 2003 ; Avelino et al., 2004 ; Bousset & Chèvre, 2012 ; Savary, 2014). Elles ont un caractère récurrent et pluriannuel puisque la survie de l'inoculum permet de faire la jonction entre deux épidémies.

1.5. Inoculum primaire et échelles d'espace : dispersion et structure de populations résidentes vs immigrantes

Prendre en compte la composante spatiale de l'environnement dans les interactions hôte-pathogènes est indispensable pour comprendre comment débute une épidémie. Avoir comme référentiel une seule parcelle sans considérer son environnement plus ou moins proche, sans distinguer un compartiment "local" d'un compartiment "distant", serait une erreur. La notion

de distance et d'origine de l'inoculum sont intimement liés à sa capacité de dispersion. Un gradient de dispersion est caractérisé par la décroissance de la quantité d'inoculum qui arrive sur un peuplement hôte en fonction de la distance d'éloignement avec la source d'émission. Cette décroissance s'explique par la dilution de l'inoculum dans le volume d'air qui croît avec la distance et par son dépôt progressif sur le sol ou sur les surfaces hôtes (Rapilly, 1991). Il est théoriquement possible de représenter des lignes isopathes (Alderman et al., 1989 ; Fernando et al., 1997) délimitant les zones où les dépôts d'inoculum ont été comparables en densité (Stedman, 1980).

Une population pathogène présente sur une parcelle, considérée comme le référentiel, sera dite locale (ou résidante), tandis qu'une population n'ayant pas été produite sur cette même unité sera dite distante (ou immigrante). Pour étudier les caractéristiques de l'inoculum primaire, on peut alors se baser sur ces termes "résidant" et "immigrant" qui, de façon intrinsèque, caractérisent son origine (Zhan et al., 1998 ; Stukenbrock et al., 2006 ; Sommerhalder et al., 2010 ; Gladieux et al., 2011).

2. Les caractéristiques de l'inoculum primaire

L'objectif de cette partie est d'illustrer la façon dont les trois facteurs mis en évidence précédemment - quantité, efficacité et origine - peuvent caractériser l'inoculum primaire. Les exemples ont été choisis en priorité parmi les champignons ascomycètes parasites de grandes cultures présents dans les zones tempérées, comme *Leptosphaeria maculans* (colza), *Rhynchosporium secalis* / *Rhynchosporium commune* (orge), *Phaeosphaeria nodorum* / *Stagonospora nodorum* (blé), *Pyrenopeziza brassicae* (colza) et *Didymella rabiei* (pois chiche). Ces parasites aux cycles de vie variés ont été largement étudiés et permettent d'avoir un aperçu de la façon dont l'inoculum primaire influence le commencement d'une épidémie.

2.1. Quantité d'inoculum

La quantité d'inoculum primaire, déterminant essentiel de la dynamique d'une maladie, est prise en compte dans les modèles de prévision et de développement des épidémies (Jones, 1998). Quantifier l'inoculum c'est lui attribuer à un moment donné une valeur numérique (quantité) : il s'agit de quelque chose de mesurable. Il existe deux approches pour quantifier l'inoculum : à la "source", c'est-à-dire là où il est produit (par exemple la quantité de spores

produites dans une parcelle par des tissus végétaux contaminés), ou au niveau de la "cible" c'est-à-dire là où il se dépose (par exemple la quantité de spores présentes dans l'air au-dessus d'une culture hôte). Le cas de résidus de culture contaminés est un bon exemple où il est possible de quantifier à la source l'inoculum primaire (Rossi et al., 2008 ; Lo-Pelzer et al., 2009 ; Landschoot et al., 2011 ; Suffert & Sache, 2011). La quantification au niveau de la cible peut se faire par piégeage biologique (Trapero-Casas et al., 1996 ; Chilvers et al., 2007 ; Maumené et al., 2012 ; Zhan et al., 2013 ; Rieux et al., 2014) ou piégeage physique (Hunter et al., 1999 ; Rogers et al., 2009 ; Duvivier et al., 2013) en utilisant par exemple des pièges à spores volumétriques. Avec l'amélioration et le perfectionnement des techniques de biologie moléculaire, leur utilisation a été couplée avec des techniques de qPCR (Schweigkofler et al., 2004 ; Fraaije et al., 2005 ; Carisse et al., 2009 ; Kaczmarek et al., 2012 ; Duvivier et al., 2013 ; Parker et al., 2014). Les dénombrements visuels, qui ont été pendant de nombreuses années la seule façon de quantifier les spores piégées (Trapero-Casas et al., 1996 ; Hunter et al., 1999 ; Bathgate & Loughman, 2001 ; Heard & West, 2014), nécessitent beaucoup de temps et d'expertise pour distinguer les différentes espèces. La corrélation entre les résultats obtenus par les deux méthodes est parfois assez faible (Fraaije et al., 2005 ; Kaczmarek et al., 2009 ; Karolewski et al., 2012 ; Piliponyte-Dzikiene et al., 2014) mais la qPCR apparaît tout de même comme l'unique solution permettant de quantifier de très faibles quantités de spores, puisqu'il s'agit d'une technique spécifique (d'une espèce), sensible (il est possible de quantifier de faibles quantités d'ADN) et rapide (lorsque l'ADN a été extrait, il est possible d'obtenir une réponse en deux ou trois heures).

2.2. Nature et efficacité de l'inoculum

L'efficacité de l'inoculum (ou efficacité d'infection), qui dépend de sa nature (type de propagule infectieuse) mais aussi de sa capacité à infecter une plante hôte plutôt qu'une autre (sensibilité variétale), est un autre déterminant de la dynamique d'une maladie.

La pathogénicité englobe à la fois les aspects quantitatifs comme la quantité de symptômes provoqués caractérisant l'agressivité (Pariaud et al., 2009 ; Zhan & McDonald, 2013) et qualitatifs (par exemple par la mesure de la virulence, c'est-à-dire la capacité de l'agent pathogène à surmonter la résistance de l'hôte et à l'infecter) de la fitness de l'agent pathogène (Tack et al., 2012). Des études récentes ont proposé des méthodes permettant de caractériser finement différentes composantes d'agressivité en conditions contrôlées sur

différents parasites fongiques du blé : rouille brune (Pariaud et al., 2007, 2009), rouille jaune (Milus et al., 2006) et septoriose (Suffert et al., 2013). La période de latence et la capacité de sporulation constituent deux composantes d'agressivité particulièrement importantes (Lannou, 2012). Chez *Z. tritici*, la capacité de sporulation peut être évaluée par la mesure de la surface maximale de lésions sporulantes sur les feuilles (Chartrain et al., 2004 ; Arraiano et al., 2006), la densité de pycnides (Eyal & Brown, 1976 ; Simon & Cordo, 1997) et le nombre de spores produites par une pycnide (Suffert et al., 2013). La période de latence (ou temps de génération) est généralement définie comme l'intervalle de temps entre l'infection et l'apparition des premières fructifications (Pariaud et al., 2009). Dans le cas de la septoriose, il s'agit du temps écoulé entre l'infection et l'apparition de la première pycnide (Shearer & Zadoks, 1972). Quand un ensemble de lésions plutôt qu'une lésion seule est considéré, ce qui est courant dans les études expérimentales, la période de latence peut aussi être estimée, à l'échelle d'une feuille, par le temps écoulé entre l'inoculation et l'apparition de la majorité des lésions sporulantes (Shaw, 1990 ; Lovell et al., 2004 ; Zearfoss et al., 2011), ou, à l'échelle d'une lésion, par le temps écoulé entre l'inoculation et l'apparition des premières pycnides (Armour et al., 2004 ; Viljanen-Rollinson et al., 2005 ; Suffert et al., 2013).

L'efficacité de l'inoculum dépend du type de propagule infectieuse. Chez les champignons ascomycètes, les spores sexuées (ascospores) sont produites dans des périthèces et sont transportées par le vent sur de longues distances, tandis que les spores asexuées (conidies ou pycnidiospores) sont produites dans des pycnides et sont souvent dispersées par la pluie, sur de courtes distances. Les différences d'efficacité d'infection et/ou de pathogénicité entre spores asexuées et sexuées ont été assez peu étudiées (Gilles et al., 2001 ; Karolewski et al., 2002 ; Li et al., 2004 ; Trapero-Casas & Kaiser, 2007). Il a été montré que le nombre minimum de spores de *Pyrenopeziza brassicae*, agent pathogène du colza, nécessaires pour provoquer une lésion sporulante sur une feuille inoculée artificiellement était d'environ 25 ascospores ou 700 pycnidiospores dans des conditions optimales (Karolewski et al., 2002). Ce résultat suggère que les ascospores de *P. brassicae* ont une efficacité d'infection plus élevée que les conidies, confirmant les résultats de Gilles et al. (2001). Chez *Didymella rabiei*, agent pathogène du pois chiche, l'inoculation de plantes avec des ascospores a montré une sévérité de la maladie significativement plus élevée que l'inoculation par des conidies chez lesquelles le processus de germination semble plus lent (Trapero-Casas & Kaiser, 2007). Enfin, chez *Leptosphaeria maculans*, les ascospores provoquent davantage de lésions que les pycnidiospores (Li et al., 2004).

2.3. Source et origine de l'inoculum

La détermination de la source principale d'inoculum primaire se fait principalement par des approches méthodologiques indirectes puisqu'il n'est pas possible de tracer toutes les spores, ni d'identifier la totalité des sources puis de les hiérarchiser entre-elles. Deux notions bien distinctes sont implicitement contenues dans les termes "source" d'inoculum et "origine". Tout d'abord, l'origine d'un agent pathogène peut se référer autant à l'origine géographique d'un inoculum (d'où provient-il ?) qu'à son origine structurale (à partir de quelle structure fongique l'inoculum a-t-il été formé ? - mycélium, sclérote, fructification sexuée ou asexuée, etc.). L'origine peut ensuite faire référence à la distance génétique entre le (ou les) isolat(s) constituant l'inoculum primaire. Boeger et al. (1993) se sont interrogés sur cette notion en étudiant des populations de *Z. tritici* (septoriose du blé) collectées en Oregon ou en Californie. Ils ont analysé la corrélation entre la distance génétique et la distance géographique, qui dépend de la capacité de dispersion de l'agent pathogène. La combinaison de l'étude de la répartition spatiale et de la diversité génotypique via des marqueurs microsatellites peut être utile pour déduire quel type d'inoculum est le plus important lors d'une épidémie (Milgroom & Peever, 2003 ; Peever *et al.*, 2004). Zwankhuizen et al (1998) ont recherché les sources d'inoculum primaire de *Phytophthora infestans*, responsable des épidémies de mildiou dans les champs de pommes de terre. Ils ont combiné une approche traditionnelle épidémiologique en étudiant les gradients de maladie et les emplacements des foyers de maladie par rapport aux sources d'inoculum potentielles (les tas de rebuts, les parcelles biologiques, des repousses en champ ou des jardins familiaux contaminés), avec une approche de génétique des populations mesurant par *DNA fingerprinting* la distribution spatiale des génotypes des agents pathogènes d'un champ infecté avec ceux collectés sur les sources d'inoculum suspectées. Ils ont établi que la principale source d'inoculum primaire était les parcelles en agriculture bio, qui contaminaient les champs de pommes de terre conventionnels adjacents. Les travaux de Shah et al. (2001) ont montré que l'inoculum primaire de *Phaeosphaeria nodorum* était présent sur les semences. Les marqueurs génétiques neutres d'isolats inoculés sur des semences ont été comparés avec ceux des isolats issus de lésions foliaires à la fin de l'épidémie, puis sur les graines collectées l'année suivante ; des profils similaires ont confirmé le rôle de la transmission par la semence.

3. Le système d'étude : *Zymoseptoria tritici*-*Triticum aestivum*

3.1. La septoriose du blé et son contrôle

Organisée autour de quatre grandes productions (blé tendre, maïs, orge, blé dur), la filière céréalière française occupe 50 % des terres arables. La France, qui produit un quart des céréales de l'Union européenne, est le quatrième producteur mondial de blé tendre (36,8 millions de tonnes en 2013 ; source FranceAgriMer). La septoriose (en anglais *Septoria tritici blotch*) est l'une des principales maladies foliaires du blé (*Triticum aestivum*). Elle est causée par le champignon ascomycète *Zymoseptoria tritici* (Quaedvlieg et al., 2011), connu jusqu'à récemment sous les deux noms binomiaux *Mycosphaerella graminicola* Fuckel (J. Schröt.) (téléomorphe - forme sexuée) et *Septoria tritici* Berk. & M.A. Curtis (anamorphe - forme asexuée). La maladie est largement répandue dans le monde, notamment en Europe de l'Ouest où les conditions climatiques tempérées sont favorables au développement du champignon ; elle peut y provoquer jusqu'à 40% de pertes de rendement (Eyal et al., 1987 ; HGCA, 2012).

En France, les méthodes de protection des cultures de blé sont principalement basées sur l'utilisation de fongicides et de variétés moins sensibles à la maladie (partiellement résistantes ou tolérantes). Certains fongicides, comme les benzimidazoles et les DMIs (inhibiteurs de la 14a-déméthylation des stérols), sont utilisés depuis les années 1970 ; d'autres, comme les QoIs (inhibiteurs externes de la quinone, appartenant à la famille des strobilurines) sont utilisés depuis la fin des années 1990 (Fraaije et al., 2005). Les benzimidazoles et les QoIs ont un seul site d'action, et une résistance à ces groupes de fongicides s'est développée dans les populations de *Z. tritici*, respectivement depuis 1984 et 2002. Les QoIs sont actuellement appliqués en mélange avec des DMIs, à raison de deux traitements en moyenne par saison culturale, afin de limiter le développement des résistances dans les populations pathogènes et d'assurer un contrôle efficace de la maladie (Fraaije et al., 2005 ; Clark, 2006 ; Jørgensen & Thygesen, 2006).

Le contexte sociopolitique et législatif français tend à favoriser une réduction de l'emploi des pesticides, notamment dans le cadre du "Projet Agro-écologie pour la France" lancé en 2012 par le ministère de l'agriculture. L'utilisation de variétés de blé résistantes (Chartrain et al., 2004) pourrait être une alternative à l'utilisation de fongicides, considérée comme une méthode couteuse, de moins en moins efficace et peu respectueuse de

l'environnement. Au cours de la dernière décennie, 18 gènes de blé (Stb1 à Stb18) conférant une résistance à *Z. tritici* ont été identifiés (Simon et al., 2012), mais aucune variété commerciale actuelle n'est totalement résistante à la septoriose. L'utilisation de gènes majeurs de résistance n'est probablement pas une solution durable à cause de la grande diversité génétique des populations de *Z. tritici* (Zhan et al., 2003 ; El Chartouni et al., 2011), qui leur confère une capacité d'adaptation élevée (Ahmed et al., 1995 ; Cowger et al., 2000 ; Zhan et al., 2002, 2006).

Une gestion agronomique raisonnée des cultures de blé (rotations, enfouissement des résidus contaminés, semis tardifs, etc.) est susceptible d'offrir des solutions pour diminuer l'incidence et la sévérité des épidémies de septoriose (Eyal et al., 1987 ; Suffert et al., 2011).

3.2. L'inoculum de *Z. tritici*, élément clé du développement d'une épidémie de septoriose

Le cycle de vie asexué de *Z. tritici*, champignon phytopathogène modèle (O'Driscoll et al., 2014), est bien connu (Figure 3). Ce parasite hémibiotrophe infecte des tissus foliaires vivants (feuilles vertes) en pénétrant par les stomates et peut survivre dans ces mêmes tissus morts ou en décomposition (chaumes et résidus). De petites nécroses foliaires portant des fructifications asexuées (pynides) apparaissent environ trois semaines après contamination, ce qui fait de la septoriose une maladie dont la période de latence est considérée comme longue. Chaque pynide est capable de produire plusieurs milliers de pycnidiospores (Eyal, 1971 ; Suffert et al., 2013) entourées d'un gel mucilagineux formant un cirrhe. Les pycnidiospores ont une forme allongée légèrement arquée, d'une longueur comprise entre 20 et 98 μm , et sont constituées de quatre à huit cellules (Figure 4 ; Sivanesan, 1990 ; Shaner, 2010). Pendant la saison culturale, la maladie se propage de plante à plante (progression horizontale) et feuille à feuille (progression verticale) sur de courtes distances par dispersion pluviale des pycnidiospores (Shaw, 1987). Lorsqu'une goutte d'eau touche un cirrhe mature, des pycnidiospores sont incorporées au sein d'un film d'eau s'étalant à la surface de la feuille ; les éclaboussures issues de la fragmentation des gouttes incidentes incorporent alors des pycnidiospores qui rejaillissent sur d'autres parties du couvert végétal et les contaminent (Rapilly, 1991). Au printemps, le développement des épidémies d'intensifie ; la vitesse de développement d'une épidémie est déterminée par le nombre de cycles de multiplication asexués emboîtés (entre quatre et six ; Agrios, 2005), qui dépend des conditions de température (Bernard et al., 2013) et du nombre d'épisodes pluvieux (Shaw & Royle,

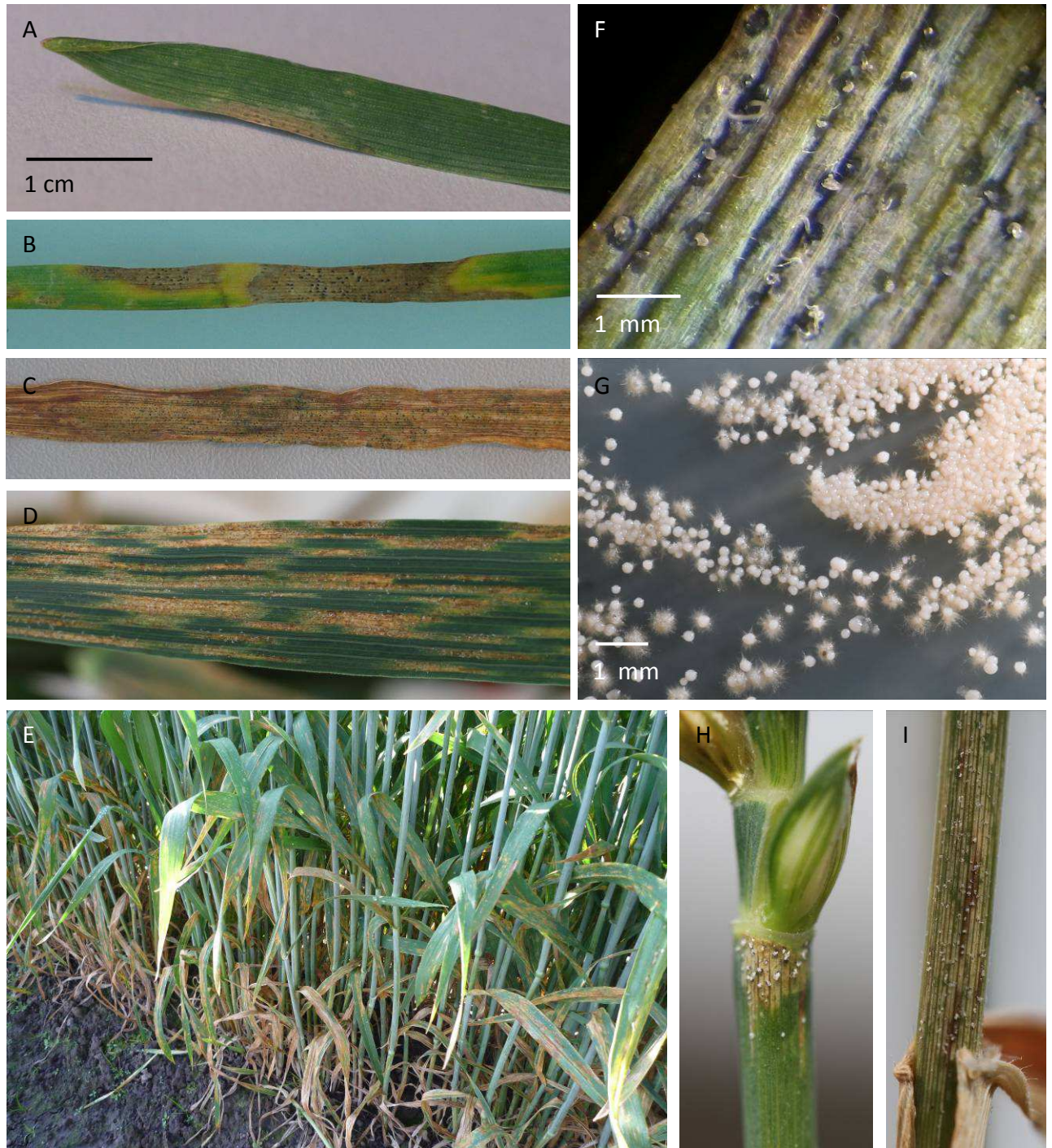


Figure 3 - La phase asexuée de *Zymoseptoria tritici*. Symptômes de septoriose sur feuilles de blé à des stades différents : (A) lésion précoce sur première feuille vraie (L1) observée au champ quatre semaines après la levée (début décembre) ; (B, C) symptômes sur feuille L1 en cours de sénescence observés au champ pendant l'hiver (fin janvier) ; (D) lésions partiellement coalescentes sur feuille antépénultième (F2) observées après inoculation artificielle en serre ; (E) attaque sévère de septoriose sur une parcelle agricole illustrée par l'état partiellement nécrosé des feuilles dans la partie basse du couvert ; (F) pycnides matures (fructifications noires) avec exsudation de cirrhes (serpentins blancs) contenant des pycnidiospores ; (G) colonies de *Z. tritici* (forme levure) obtenues après six jours de culture sur milieu PDA ; (H) pycnides et cirrhes situées sous l'épi à la base du rachis et (I) sur tige après inoculation artificielle (photos F. Suffert).

1993). La progression de la maladie se faisant du bas vers le haut du couvert, la capacité du blé à émettre de nouvelles feuilles plus rapidement avant que le parasite ne puisse les infecter (phyllochrone vs. pathochrone,) détermine généralement la capacité du couvert à "échapper" naturellement à la maladie ; dans le cas contraire, il est nécessaire de procéder à des traitements fongicides. Le pathochrone correspond au nombre de phyllochrones (durée entre l'apparition de deux feuilles successives) par période de latence. Le nombre d'étages foliaires qui émergent pendant une période de latence varie entre 1,7 et 2,9 en fonction de la température moyenne (Lovell et al., 1997).

La forme sexuée du champignon (ascospores) a pour la première fois été décrite en Nouvelle-Zélande (Sanderson, 1972). Depuis, elle a été identifiée sur tous les continents, en Australie (Brown, 1975), aux Etats-Unis (Garcia & Marshall, 1992), au Chili (Madariaga, 1986), au Royaume-Uni (Scott et al., 1988), aux Pays-Bas (Kema et al., 1996) et en France (Halama, 1996 ; Suffert & Sache, 2011). Les ascospores issues de la reproduction sexuée sont considérées comme étant la principale forme d'inoculum primaire (Figure 5). Elles se forment sur des débris de la culture de blé de la saison n-1 (Brown et al., 1978 ; Shaw & Royle, 1989 ; Suffert et al., 2011) et contaminent le blé de la saison n dès la levée (Suffert & Sache, 2011), de la fin de l'automne à la fin de l'hiver. Dans l'hémisphère nord, cette période correspond généralement au pic de production des ascospores (Shaw & Royle, 1989 ; Hunter et al., 1999). Contrairement aux pycnidiospores, les ascospores sont dispersées par le vent sur de longues distances. Elles sont produites dans des fructifications, les périthèces, qui résultent de la rencontre de deux individus de type sexuel opposé (Mat1-1 et Mat1-2 ; Kema et al., 1996 ; Waalwijk et al., 2002) et peuvent chacun libérer jusqu'à 200 ascospores (Eriksen & Munk, 2003). Les ascospores ont une forme ovoïde et sont bicellulaires ; leur longueur est comprise entre 10 et 15 μm et leur largeur entre 2,5 et 3,0 μm (Sivanesan 1990 ; Garcia & Marshall, 1992). Si *Z. tritici* peut être cultivé *in vitro*, par exemple sur milieu PDA (potato dextrose agar) où il revêt une forme levure (bourgeoisement végétatif conidien), le croisement de deux isolats reste difficile en conditions semi-contrôlées (Kema et al., 1996 ; Suffert et al., non pub.).

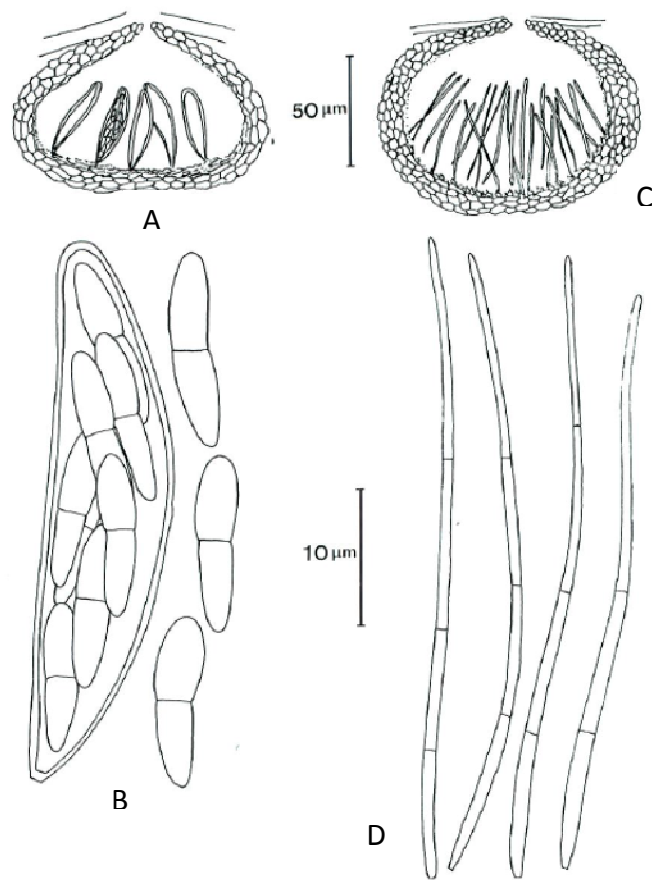


Figure 4 - Les deux types de spores de *Zymoseptoria tritici*. (A) Section verticale d'un périthèce ; (B) asque et ascospores ; (C) section verticale d'une pycnide ; (D) pycnidiospores (d'après Sivanesan, 1990).

On a longtemps pensé que les pycnidiospores (forme asexuée) constituaient la seule source d'inoculum primaire de *Z. tritici* (Weber, 1922 ; Brokenshire, 1975). Il est désormais acquis que les infections primaires sont provoquées par les ascospores, potentiellement transportées sur de longues distances par le vent, et que les infections secondaires sont provoquées par les pycnidiospores transportées par la pluie sur de courtes distances (Figure 6). Une revue de synthèse consacrée aux phases précoces de la septoriose (Suffert et al., 2011) précise toutefois que les mécanismes qui conditionnent le développement d'une épidémie sont plus nombreux et interagissent de façon complexe. Les hypothèses que plusieurs espèces de graminées sauvages, des semences de blé contaminées, ou des repousses de blé malades constituent une source d'inoculum primaire ne peuvent ainsi être totalement exclues.

Les pycnidiospores présentes sur résidus de blé constituent une source d'inoculum potentielle non négligeable, qui ne concerne cependant que la succession de deux cultures de blé *in situ* (monoculture), compte tenu de la très courte distance de dissémination de ce type de spores (Holmes & Colhoun, 1975 ; Shaw, 1987). On peut ainsi faire l'hypothèse qu'une parcelle en monoculture de blé depuis plusieurs années et semée en direct rend possible la subsistance d'une quantité non négligeable de génotypes de *Z. tritici* se multipliant plusieurs années à la suite par cycles asexués, en plus des recombinants issus de la reproduction sexuée (Zhan et al., 1998 ; Zhan & McDonald, 2013).

Alors que les épidémies de septoriose s'intensifient généralement entre mars et juin par multiplication asexuée (pycnidiospores), des contaminations considérées comme des infections secondaires peuvent être provoquées à la fin du printemps par des ascospores produites à partir des débris des cultures de blé précédentes (présents sur le sol de parcelles distantes) ou sur des feuilles sénescents de la culture de l'année en cours devenues le siège d'une reproduction sexuée précoce (Hunter et al., 1999 ; Clinckemaillie et al., 2010 ; Duvivier et al., 2013). Cette hypothèse est cohérente avec l'occurrence de pics d'ascospores dans l'air en fin de saison culturale (Hunter et al., 1999 ; Bathgate & Loughman, 2001 ; Duvivier et al., 2013). Des observations au champ réalisées début juin attestent de la présence de lésions sur les feuilles supérieures F1 et F3-F4, mais de leur absence sur F2 (C. Maumené, Arvalis-Institut du Végétal, Boigneville, com. pers.), qui pourraient illustrer les conséquences de ce phénomène.

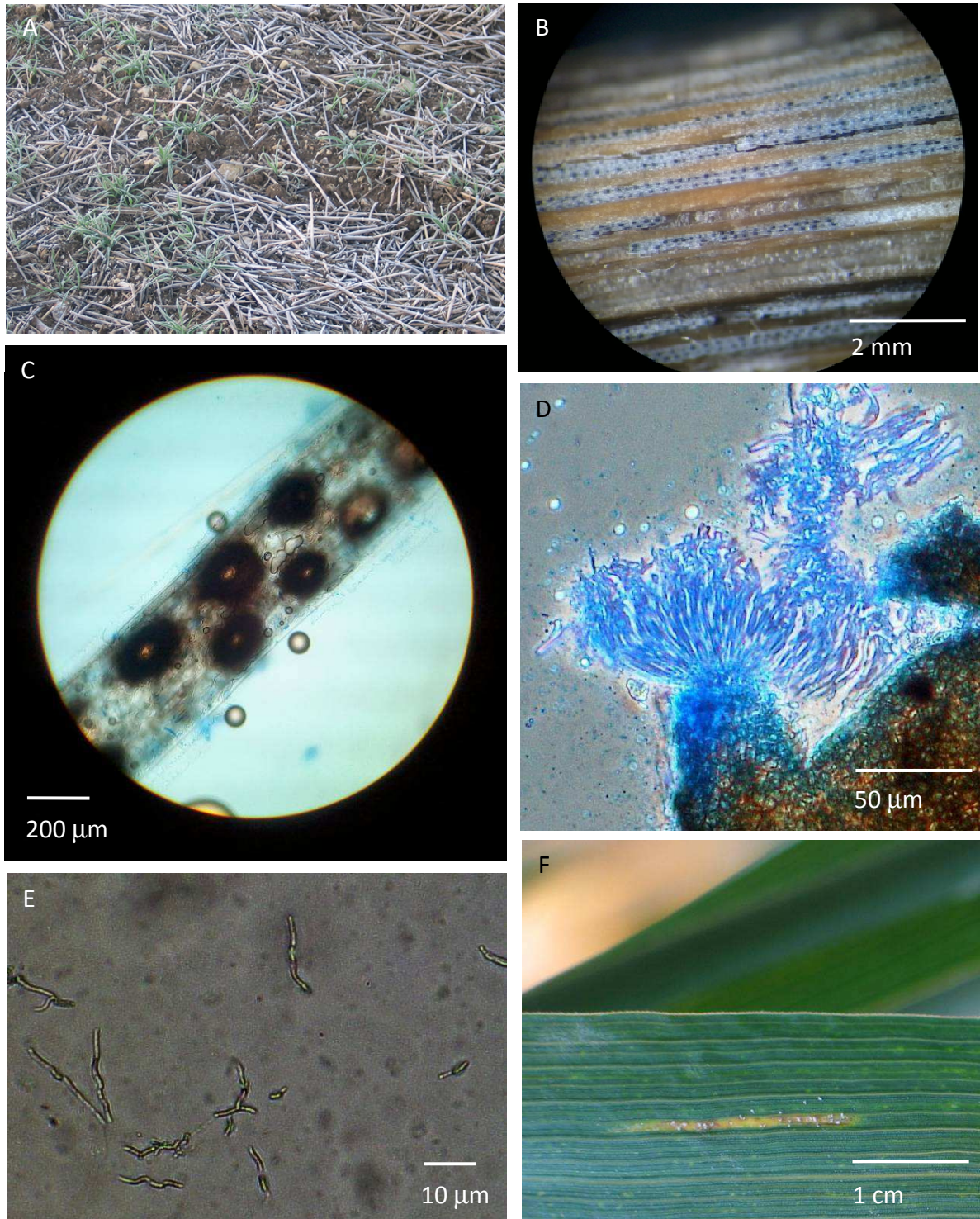


Figure 5 - La phase sexuée de *Zymoseptoria tritici*. (A) Résidus de blé présents sur le sol de la parcelle expérimentale suivie pendant la thèse (siège de la reproduction sexuée et source d'inoculum primaire) ; (B) paille de blé contaminée observée à la loupe binoculaire sur laquelle sont visibles des alignement de périthèces (fructifications noires) implantés dans les espaces inter-nervaires ; (C) alignement de périthèces observés au microscope optique ; (D) périthèce (marron) et asques contenant des ascospores (bleu) présents sur des résidus de blé observés au microscope optique ; (E) hyphes mycéliens issus de la germination d'ascospores sur milieu PDA ; (F) lésion sporulante sur feuille drapeau (F1) apparue trois semaines environ après inoculation artificielle par des ascospores (photos F. Suffert).

Compte tenu de ce qui précède, représenter une épidémie annuelle comme une série de cycles de reproduction asexués succédant à un seul cycle de reproduction sexuée serait simpliste. Les contaminations par les ascospores et par les pycnidiospores peuvent intervenir simultanément à différents stades de l'épidémie. On ne peut leur assigner les rôles exclusifs d'inoculum primaire et d'inoculum secondaire, respectivement ; et chacun des deux types d'inoculum est mobilisé de façon différente selon les échelles de temps et d'espace considérées. Les processus de contamination se combinent et varient en intensité selon les années, d'où l'intérêt, lorsque c'est possible, de raisonner sur une "succession d'épidémies" et de prendre en considération ce qui se passe pendant la période inter-épidémique.

3.3. Quelques méthodes permettant d'étudier l'inoculum primaire de *Z. tritici* : état de l'art

3.3.1. Couplage entre piégeage de spores et quantification d'ADN fongique

Des méthodes de couplage entre piégeage d'ascospores de *Z. tritici* dans l'air (Hunter et al., 1999 ; Bathgate & Loughman 2001) et quantification d'ADN fongique ont déjà été réalisés avec succès, soit pour suivre les quantités d'inoculum aérien au cours d'une saison culturale (Duvivier et al., 2013), soit pour suivre la présence de gènes de résistance à des fongicides dans les populations (Fraaije et al., 2005). Le dénombrement visuel au microscope optique des ascospores piégées ne semble pas adapté à la quantification des ascospores de *Z. tritici*. Celles-ci sont en effet de forme ovoïde et ressemblent aux ascospores d'autres espèces fongiques potentiellement présentes au-dessus des parcelles de blé du Bassin Parisien : spores de *Didymella exitialis* (sur blé), *Pyrenophora tritici-repentis* (sur blé), *Septoria passerini* (sur orge), *Stagonospora nodorum* ou *Stagonospora avenae* f. sp. *tritici* (sur blé, orge et triticales), *Mycosphaerella pinodes* (sur pois). La quantification de l'inoculum aérien par une technique de quantification de l'ADN comme la PCR quantitative (qPCR), plus rapide, spécifique, sensible et déjà largement répandue, est préférable (West et al., 2008).

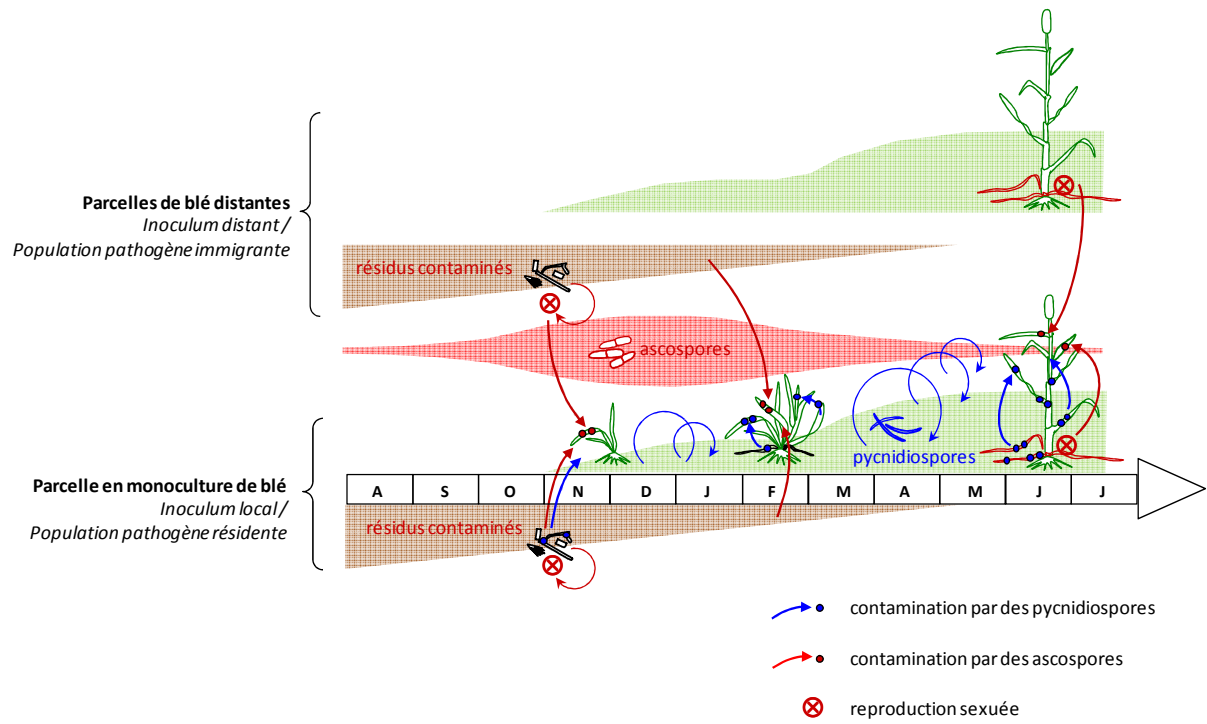


Figure 6 - Représentation schématique de la dynamique annuelle d'une épidémie de septoriose, illustrant l'influence des principales sources d'inoculum primaire et sa nature (spores asexuées et sexuées).

3.3.2. Expérimentations de type *mark-release-recapture*

Les expérimentations de type *mark-release-recapture* ont été développées assez récemment sur des populations de *Z. tritici* (Zhan et al., 1998, 2001, 2002 ; Zhan & McDonald, 2013). Elles permettent de suivre le développement d'individus "marqués" (dont on connaît certaines des caractéristiques génotypiques - marqueurs RFLP, SSR ou SNPs rares - pour pouvoir les identifier) et utilisés pour contaminer artificiellement une ou plusieurs parcelles de blé. Il est ensuite possible de déterminer, au cours ou à la fin de l'épidémie, la proportion d'individus ayant été introduits artificiellement, celle d'individus recombinants et celle d'individus immigrants. Ce type d'expérimentation convient particulièrement bien à l'étude de maladies causées par des champignons ayant un inoculum primaire aérien comme *Z. tritici* (Zhan & McDonald, 2004 ; Zhan et al., 1998 ; 2013) ou *S. nodorum* (Bennett et al., 2007 ; Sommerhalder et al., 2010 ; 2011). L'inconvénient majeur de cette approche est qu'il n'est possible d'inoculer qu'un nombre réduit d'isolats, au regard de la très grande diversité des populations naturelles.

3.3.3. Génétique des populations : marqueurs neutres, échelles larges

La génétique des populations permet de comprendre les processus évolutifs impliqués dans la création et le maintien de la variation génétique au sein et entre des populations, en analysant les fréquences alléliques. Les dimensions spatiales et temporelles sont omniprésentes dans les concepts de génétique des populations que sont la structure de la population, les déterminants des systèmes de reproduction (*mating-type* pour la reproduction sexuée), la recombinaison et la migration (ou flux de gènes entre les populations). L'apport de la génétique des populations à l'épidémiologie a été décrit par Milgroom & Peever (2003). Une comparaison plus étroite des deux disciplines montre que de nombreux concepts sont effectivement partagés (démogénétique, épidémiologie moléculaire), bien que le vocabulaire soit différent. Par exemple, les concepts épidémiologiques "dispersion" et "inoculum primaire" rejoignent les concepts de "migration" (ou flux de gènes) et "population fondatrice" utilisés par les généticiens des populations. Les deux disciplines posent des questions similaires au sein de cadres conceptuels différents. En employant différents outils il est possible d'arriver à des conclusions similaires et/ou complémentaires. Dans l'exemple de la dispersion/migration, un épidémiologiste recueillera des données sur la densité de spores, le gradient de maladie, sa configuration spatiale, etc., tandis que le généticien des populations analysera la diversité

génétique, les fréquences alléliques de certains gènes d'intérêt, etc. L'utilisation de marqueurs moléculaires neutres peut fournir une bonne évaluation de la structure des populations constituant l'inoculum primaire, mais ne renseigne pas sur leur différences de profil de virulence (pathotypes) ou d'agressivité. Idéalement, au moins deux approches doivent être combinées (Milgroom & Peever, 2003).

L'analyse de la diversité génétique et de la structure des populations de *Z. tritici* a majoritairement été réalisée à des dates uniques et à une large échelle géographique sur plusieurs continents (Zhan et al., 2002, 2003 ; Razavi & Hughes, 2004 ; Abrinbana et al., 2010 ; El Chartouni et al., 2011; Boukef et al., 2012), moins à une échelle locale (champ ou plante-feuille, McDonald & Martinez, 1990 ; Chen et al., 1994 ; Linde et al., 2002 ; El Chartouni et al., 2012). La grande majorité des populations collectées et étudiées à travers le monde depuis deux décennies (par analyses AFLP, RFLP ou SSR) présentent une diversité génétique très élevée et aucune structure à l'échelle continentale ou régionale (Schnieder et al., 2001 ; Linde et al., 2002 ; Drabesova et al., 2012), à de rares exceptions près (Abrinbana et al., 2010 ; El Chartouni et al., 2011). Les populations de *Z. tritici* résultent d'une reproduction sexuée très intense (Chen & McDonald, 1996 ; Zhan et al., 1998 ; 2002) favorisant les flux de gènes (Boeger et al., 1993 ; Zhan & McDonald, 2004) et l'apparition rapide de résistances aux fongicides (Zhan et al., 2006 ; Leroux *et al.*, 2006 ; Drabesova et al., 2012 ; Boukef et al., 2012). A titre de comparaison, chez des espèces ascomycètes ayant une biologie proche de *Z. tritici* comme *Ascochyta rabiei* ou *Phaeosphaeria nodorum*, une forte diversité génétique a été retrouvée dans des populations géographiquement très éloignées mais présentent des taux de différenciation parfois plus élevés (F_{ST} proche de 0,2 chez *A. rabiei* et inférieur à 0,05 chez *Z. tritici* et *P. nodorum* ; Peever et al., 2004). La date d'échantillonnage des populations de *Z. tritici* a rarement été considérée comme un facteur qui pourrait biaiser la caractérisation de leur structure génétique, malgré des différences de quantité ou de dispersion entre chaque type d'inoculum (sexué et asexué) selon la période épidémique.

3.3.4. Travaux sur les fongicides et les résistances variétales : marqueurs de traits soumis à sélection

Des marqueurs de traits soumis à pression de sélection, notamment ceux impliqués dans les résistances aux fongicides et le contournement des résistances variétales, ont été largement étudiés. Etudier la résistance aux fongicides dans les populations de *Z. tritici* permet de mieux

comprendre les processus de sélection impliqués dans leur généralisation (Zhan et al., 2006 ; Drabesova et al., 2012 ; Boukef et al., 2012). La structuration des populations de *Z. tritici* par les variétés de blé a été mise en évidence à plusieurs reprises, notamment sur deux aspects, le niveau de résistance du cultivar à l'agent pathogène et la variété d'origine des isolats. Les populations collectées sur des cultivars de blé sensibles à la septoriose produisaient des niveaux de maladie plus élevés que des populations isolées à partir de cultivars résistants ou moyennement résistants (Ahmed *et al.*, 1996 ; Zhan & McDonald, 2013) ; les isolats étaient globalement plus agressifs sur les cultivars dont ils étaient issus (sympatriques) que sur d'autres cultivars (allopatriques). Le piégeage des spores, combiné avec la qPCR a été employé pour quantifier dans l'inoculum aérien la présence de gènes d'avirulence (Van de Wouw et al., 2010) ou suivre des gènes conférant une résistance aux fongicides (Fraaije et al., 2005). Ce type d'approche a notamment permis de montrer que dans une sporée aérienne, la proportion d'individus résistant aux QoI augmentait dans des proportions significatives après un traitement fongicide en cours de culture (35 à 80% après une première application et 90% après une seconde ; Fraaije et al. 2005). Après la récolte, la fréquence des allèles résistants est redescendue à 60%, suggérant l'arrivée d'ascospores provenant d'autres sources d'inoculum distantes. L'étude de la fréquence de résistance aux fongicides dans les populations de *Z. tritici*, à condition que celle-ci ne soit pas généralisée, pourrait être utilisée comme marqueur et contribuer à la détermination de l'origine de l'inoculum primaire.

4. Objectifs, stratégie et plan de la thèse

Les maladies foliaires fongiques sont une contrainte majeure des systèmes céréaliers. Elles constituent par ailleurs de précieux modèles d'étude pour comprendre comment commence une épidémie, questionnement récurrent en épidémiologie végétale. La thèse a eu pour objectif d'étudier les déterminants des phases précoces des épidémies de septoriose du blé et notamment de répondre à la question "Comment commence une épidémie à l'échelle d'une parcelle ?" Tenter de répondre à une question à la fois simple et large a nécessité de s'intéresser à l'ensemble des éléments qui conditionnent le "commencement" d'une épidémie : quels processus épidémiques interviennent, comment ces processus se combinent, par quels



Figure 7 - Dispositif pluriannuel au champ (Grignon) utilisé comme cadre expérimental de la thèse. (A) Parcelle cultivée en monoculture de blé à la fin de l'été, quelques semaines après la moisson après broyage des résidus, puis (B) au début et (C) à la fin de l'hiver ; (D) séance de diagnostic permettant d'estimer la sévérité d'attaque de septoriose sur plantule de blé collectée au champ ; (E) piège volumétrique (Burkard) utilisé pour collecter les ascospores présentes dans l'air au dessus de la parcelle expérimentale ; (F) boîte de tubes cryogéniques permettant de conserver à -80°C les isolats de *Z. tritici* collectés au champ (photos F. Suffert).

facteurs sont-ils influencés et à quelles échelles de temps et d'espace ? Dans cette étude, je me suis positionné à une échelle locale, sur une courte période : deux épidémies annuelles consécutives dans deux parcelles de blé proches l'une de l'autre (Figure 7). Le cadre expérimental a consisté en un dispositif pluriannuel situé sur le domaine de Grignon (78). Une première parcelle, caractérisée par l'absence de résidus (implantation différente chaque année après une culture autre que du blé), a été exposée à des sources d'inoculum primaire distantes (population pathogène considérée comme immigrante). Sur une seconde parcelle, en monoculture de blé depuis 2007, était présente une quantité importante de résidus de blé contaminés agissant comme source locale d'inoculum primaire (population pathogène considérée comme résidante).

La stratégie globale que j'ai adoptée a été la suivante : 1. Identifier les principaux déterminants des phases épidémiques précoces à caractériser (quantité, efficacité et origine de l'inoculum primaire) ; 2. Mobiliser les concepts de plusieurs disciplines et combiner différentes approches méthodologiques (épidémiologie quantitative, biologie moléculaire, génétique des populations, expression des interactions hôte-pathogène) pour répondre à des questions ciblées et appliquées à un seul et même cas d'étude expérimental (Figure 8) ; 3. Analyser séparément les résultats obtenus et répondre indépendamment aux différentes questions (chapitres II, III, IVa et IVb) ; 4. Identifier les points de convergence (ou de divergence) entre les résultats obtenus et discuter la complémentarité des différentes approches.

L'implication relative des deux types de spores de *Z. tritici* (pycnidiospores et ascospores) dans le commencement d'une épidémie a déjà été analysée dans sa globalité (Suffert et al., 2011) puis testée, plus spécifiquement, sur le même dispositif expérimental (résidus dans une parcelle en monoculture de blé considérés comme une "source" locale d'inoculum ; Suffert & Sache, 2011). Cette première étape a confirmé que les ascospores constituaient la principale forme d'inoculum primaire. S'appuyant sur ce résultat, l'objectif du **deuxième chapitre** était de quantifier cet inoculum primaire en début d'épidémie (Figure 7A-C), en s'intéressant cette fois-ci non pas à la quantité d'ascospores que la "source" était capable d'émettre, mais à la quantité d'ascospores disponibles au-dessus de la parcelle de blé considérée comme une "cible", sans forcément pouvoir en distinguer l'origine (locale vs. distante). Plusieurs questions ont abordées dans ce chapitre : quelles quantités d'ascospores présentes dans l'air peuvent être détectées ou quantifiées ? Comment ces quantités varient-elles entre le début et la fin d'une épidémie et entre deux années ? Quels sont les effets de la

présence locale de résidus sur cette quantité ? Quelle est l'influence de cette quantité sur la précocité des épidémies (Figure 7D) ? La quantité d'ascospores présente dans l'air au-dessus de chacune des deux parcelles a été suivie très précocement pendant les saisons 2011-2012 et 2012-2013 en combinant piégeage de spores (piège volumétrique Burkard ; Figure 7E) et quantification de leur ADN (par qPCR, méthode initialement développée par Duvivier et al. (2013)). Les limites techniques de la méthode, en particulier les seuils de détection et de quantification, ont été déterminées et discutées.

L'objectif du **troisième chapitre** était de comparer l'efficacité des deux types de spores (ascospores et pycnidiospores) constituant l'inoculum primaire et secondaire de *Z. tritici*. Y a-t-il des différences de pathogénicité entre ascospores et pycnidiospores ? Le cas échéant, quelles pourraient être les causes et les conséquences de ces différences pendant les phases de l'épidémie où les deux types de spores jouent un rôle ? Pour répondre à ces questions, j'ai inoculé en serre des feuilles de plants de blé adultes en utilisant successivement des ascospores et des pycnidiospores. Dans un premier temps, des ascospores collectées à partir de débris contaminées ont été utilisées. Le développement des lésions a été suivi et les composantes d'agressivité (sporulation et période de latence) ont été estimées en s'inspirant de la méthode de Suffert et al. (2013). Dans un second temps, des pycnidiospores produites sur les lésions issues de l'inoculation par les ascospores ont été collectées et utilisées pour inoculer les feuilles d'un second lot de plantes adultes. La comparaison entre les deux types de spores a donc pu porter sur des individus génotypiquement identiques. Cet élément essentiel de la stratégie a rendu la comparaison de la pathogénicité des deux types de spores plus robuste que certaines études précédentes (par exemple, Karolewski et al., 2002).

L'objectif du **quatrième chapitre** était d'inférer l'origine de l'inoculum impliqué dans les stades précoces d'une épidémie. Pour répondre à cet objectif j'ai développé deux approches indépendantes, supposées produire des résultats complémentaires. La première approche a été basée sur l'analyse des changements dans la structure génétique (marqueurs neutres SSR) susceptibles d'être mis en évidence dans les populations pathogènes à une échelle fine. La seconde approche a été basée sur la comparaison du profil d'agressivité de certaines de ces populations en exploitant leur adaptation différentielle à leur variété hôte d'origine.

Dans la **première partie du quatrième chapitre** l'objectif était de déterminer si les changements dans la structure d'une population et sa différenciation, généralement très faible chez *Z. tritici* quelle que soit l'échelle considérée (Linde et al., 2002), est perceptible à une

échelle très fine en fonction des périodes épidémiques (début vs. fin), du type d'inoculum majoritairement impliqué (ascospores vs. pycnidiospores) et de son origine suspectée (locale vs. distante). Nous avons choisi de baser notre approche sur l'analyse du polymorphisme de séquences microsatellites SSR (séquences d'ADN constituées d'une répétition de motifs nucléotidiques dont les longueurs varient selon les individus), méthodologiquement proche de celle développée par Chen et al. (1994). Nous avons utilisé le panel de marqueurs SSR mis au point par Gautier et al. (2014). Les questions suivantes ont été abordées : localement, la structure des populations de *Z. tritici* change-t-elle au cours du temps, notamment entre le début et la fin d'une épidémie ? Le cas échéant, quels facteurs peuvent expliquer des différenciations, même minimales, entre populations ? Sur la parcelle en monoculture de blé, arrive-t-on à identifier une continuité entre la population résidente à la fin d'une épidémie et la population résidente au début de l'épidémie de la saison suivante ? Les analyses génétiques ont porté sur un total de 800 isolats collectés pendant trois saisons épidémiques successives dans les deux parcelles du dispositif pluriannuel (Figure 7F). Les effets type de spores (ascospores vs. pycnidiospores), année (2010-2011, 2011-2012 et 2012-2013), période épidémique (début vs. fin) et proximité à la source locale d'inoculum (parcelle avec résidus ou sans résidus) ont été estimés et discutés.

Dans **la seconde partie du quatrième chapitre** l'objectif était d'inférer l'origine d'une population locale en comparant son profil d'agressivité (traits a priori sélectionnés et caractéristiques de l'interaction isolat de *Z. tritici*-variété de blé) à celui de populations dont l'origine (locale vs. distante) était connue. Les questions suivantes ont été abordées : peut-on mettre en évidence une adaptation différentielle des populations à leur variété sympatrique (variété sur laquelle elles ont été collectées) par rapport à une variété allopatrique ? Le cas échéant, la comparaison des profils d'agressivité de différentes populations permet-elle d'identifier l'origine de certaines d'entre-elles ? Le profil d'agressivité (sporulation et période de latence) de la population collectée sur plantules en début d'épidémie (fin 2009) dans la parcelle en monoculture de blé (présence de résidus contaminés) a été comparé à celui des populations considérées comme résidente et immigrante.

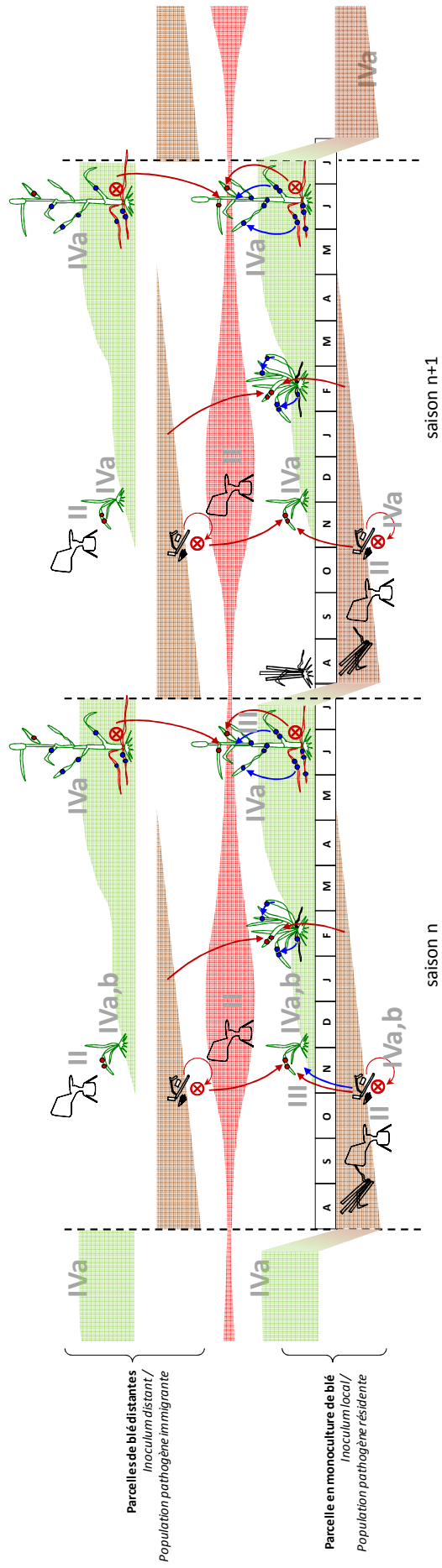


Figure 8 - Représentation schématique de la dynamique d'une épidémie de septoriose à une échelle pluriannuelle. Les objectifs et les questions abordés dans les différents chapitres de la thèse (II, III, IVa et IVb) sont positionnés au niveau des processus auxquels ils font référence.

Chapitre II

Disponibilité locale d'ascospores de *Z. tritici* et précocité des épidémies de septoriose

Is onset of *Septoria tritici* blotch epidemics related to local availability of ascospores? *

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Abstract

To elucidate the early epidemic stages of *Septoria tritici* blotch, we concomitantly assessed at a small spatiotemporal scale over two years the early disease dynamics and airborne concentration in *Zymoseptoria tritici* ascospores, using spore traps coupled with a qPCR assay. One plot, with the crop debris left, provided a local source of primary inoculum, while the other one, without debris, lacked any. The limits of detection of the assay were 13 ± 4 ascospores.m⁻³.day⁻¹ (lower limit) and 19 ± 6 ascospores.m⁻³.day⁻¹ (upper limit), while the limit of quantification was 121 ± 40 ascospores.m⁻³.day⁻¹. According to those limits, daily spore trap samples were classified as detectable, quantifiable, not detectable or not quantifiable. Proportion of samples assigned to the different classes and numbers of spores in samples classified as quantifiable were significantly different between years, time periods (from November to March) and spore trap location (field with or without debris). The effect of year on the airborne ascospore concentration was high: 22 daily peaks with more than 230 ascospores.m⁻³.day⁻¹ were identified in autumn 2012-2013, but none in autumn 2011-2012. The local presence of wheat debris had no obvious effect on the amount of airborne ascospores or on the earliness of the two epidemics in the field with debris, especially the year with high inoculum pressure (2012-2013). These results suggest that the amount of primary airborne inoculum available over a wheat crop is not a limiting factor for the epidemic onset.

Keywords

Debris management, limit of detection, limit of quantification, *Mycosphaerella graminicola*, primary inoculum, qPCR, spore trap, wheat.

Introduction

The ascomycete fungus *Zymoseptoria tritici* (Quaedvlieg et al., 2011), also known as *Septoria tritici* (teleomorph, *Mycosphaerella graminicola*), causes Septoria tritici blotch, a worldwide foliar disease of wheat. In England, Septoria tritici blotch is the most damaging disease of wheat, causing yield losses of 30 to 50% (HGCA, 2012). Wind-dispersed ascospores resulting from the sexual reproduction of the fungus on infected wheat debris are considered as the main source of primary inoculum (Shaw & Royle, 1989; Bathgate & Loughman, 2001; Suffert et al., 2011). In fungi surviving on infested residues, cropping practices aimed at reducing the amount of primary inoculum will generally reduce the subsequent disease intensity and are often recommended as a method of disease control (Eyal et al., 1987); the quantitative test of this assumption requires the accurate quantification of the amount of ascospores acting as the primary inoculum. In tan spot disease of wheat, disease severity was positively correlated with the initial amount of ascospores of *Pyrenophora tritici-repentis* released by residue (Adee & Pfender, 1989). A similar relationship was recorded in barley leaf blotch, caused by *Rhynchosporium secalis* (Fountaine et al., 2010), in ascochyta blight of chickpea, caused by *Mycosphaerella rabiei* (Trapero-Casas et al., 1996), in brown spot of pear, caused by *Stemphylium vesicarium* (Rossi et al., 2008), and in South American leaf blight of rubber, caused by *Microcyclus ulei* (Guyot et al., 2014); in the latter case, the nature of the ascospore source is unknown yet, though. In Septoria tritici blotch, the timing of ascospore release appears to influence the earliness of epidemics: disease developed earlier in a wheat field where infected debris from the previous wheat crop had been left than in a neighbouring field from which debris had been removed (Suffert & Satche, 2011). The experiment was not fully conclusive since the amount of airborne ascospores was not quantified.

Airborne spores can be routinely captured by high throughput spore traps (West et al., 2008; Jackson & Bayliss, 2011). The direct counting of the spores on trapping slides or tapes under a microscope can be performed only for spores that can be unambiguously assigned to a fungal species. This is, unfortunately, not possible when spores lacking specific morphological features, such as ascospores of *Z. tritici*, are caught together with spores of other fungal species similar in shape and size. Methods integrating spore trapping with qPCR assays were developed to quantify DNA extracted from trapped ascospores of the *Leptosphaeria maculans* / *L. biglobosa* complex (Kaczmarek et al., 2009; 2012; Piliponyte-

Dzikiene et al., 2014), *Pyrenopeziza brassicae* (Karolewski et al., 2012), and *Sclerotinia sclerotiorum* (Rogers et al., 2009; Parker et al., 2014). In these studies, the quantification of airborne inoculum could not be correlated with disease intensity. In *Z. tritici*, qPCR assays were used to monitor fungicide resistance (Fraaije et al., 2005) and the spatio-temporal distribution of inoculum (Duvivier et al., 2013), without specific emphasis on the onset of epidemics.

The objective of this study was to assess the influence of the amount of airborne ascospores of *Z. tritici* on the earliness of Septoria tritici blotch. To this end, the incidence and severity of disease were recorded for two consecutive growing seasons, in two neighbouring wheat plots, the one with infected debris left to provide a local source of ascospores and the other with no debris. The DNA content of ascospores caught either using a spore trap or produced by infected wheat debris was quantified using the specific qPCR assay proposed by Duvivier et al. (2013), taking into account the technical limits of the qPCR assay when very small amounts of spores are to be quantified.

Materials and methods

Experimental field design

A field experiment derived from that described in Suffert & Sache (2011) was carried out during two successive growing seasons (2011-2012 and 2012-2013, hereafter referred to the first and second season, respectively) at the Grignon experimental station (France; 48°51'N, 1°58'E). Two plots were sown on 17 October 2011 and 16 October 2012, respectively, with wheat cv. Soissons, moderately susceptible to Septoria tritici blotch (rated 5 on a 1-10 scale of decreasing susceptibility, Arvalis Institut du Végétal-CTPS), without fungicide application. In the first plot (D+, 20 m × 100 m), cropped with wheat since 2007, straw was chopped at harvest (mid July) and left on the soil surface; debris were chopped and partially buried to a depth of 10 cm six weeks later (late September). Debris naturally contaminated with *Z. tritici* was expected to act in autumn as a local source of primary inoculum. The second plot (D₀, 40 m × 100 m), located 300 m apart from plot D+, did not contain any wheat debris since it had been grown the previous season with faba bean.

Table 1 - Features of the experimental field design.

2011-2012 (first season)	debris management ^a	
	D+	D ₀
preceding crop	wheat	faba bean
sowing date	17/10/2011	17/10/2011
seedling emergence date	01/11/2011	01/11/2011
debris density (g.m ⁻² , on 11/01/2012)	238	0
plant density (plant.m ⁻² , on 11/01/2012)	152	154

2012-2013 (second season)	debris management ^a	
	D+	D ₀
preceding crop	wheat	faba bean
sowing date	16/10/2012	15/10/2012
seedling emergence date	22/10/2012	22/10/2012
debris density (g.m ⁻² , on 11/12/2012)	470	0
plant density (plant.m ⁻² , on 19/03/2013)	114	140

^a D+ = wheat debris left; D₀ = faba bean debris (traces) and no wheat debris

Air temperature at a height of 2 m above soil level and rainfall were recorded hourly by an automatic weather station (Enerco 516i, Cimel Électronique, France) located c. 500 m from the plots. The thermal time t , expressed in degree-days, was calculated, starting from the sowing date, by summing the daily mean air temperature using a 0°C base temperature.

Disease assessment

Disease was assessed at 12 sampling dates in the first season and 10 sampling dates in the second one, twice or thrice monthly at the beginning of the epidemics (autumn and winter) and thereafter monthly during the epidemic phase (spring) until May. At each sampling date, five plants were randomly collected in five 1-m² quadrats per plot, located 20 meters away from each other, and washed from soil particles. Disease severity and incidence were calculated on the main stem and the first tiller of each plant sample. The leaf layers were identified according to their order of emergence (L1 is the first leaf, L2 the second, etc.). For a given layer, disease incidence was estimated as the percentage of leaves with visible lesions (Suffert et al., 2013). Severity was estimated as the average percentage of necrotic leaf area covered by pycnidia using a diagrammatic scale (1, 2, 3 and 5%, then 10, 15, 20% until 100%).

Quantification of ascospore production from infected wheat debris

Infected wheat debris were collected on the plot D+ at five sampling dates (10 October 2012, 12 November 2012, 7 December 2012, 9 January 2013 and 4 March 2013) during the early stages of the epidemics. Debris were cut in 2-cm fragments and kept at 18°C during 24 h. Ten g of debris were evenly spread on a wet filter in a moist box (Suffert & Sache, 2011) in order to promote the ejection of ascospores from mature pseudothecia. In each box, eight Petri dishes containing PDA medium (39 g.L⁻¹) were placed upside down 1 cm above the debris. The boxes were placed for 6 hours at 18°C in the dark; thereafter Petri dishes were closed and incubated in the same conditions. Germination occurred few hours later but the resulting colonies were visually counted five days later, assuming that a colony resulted from the germination of a single ascospore (Suffert & Sache, 2011). The amount of ascospores produced per m² in each field plot was so estimated for each date by dividing the amount of ascospores produced per g of debris by the density of debris (Table 1).

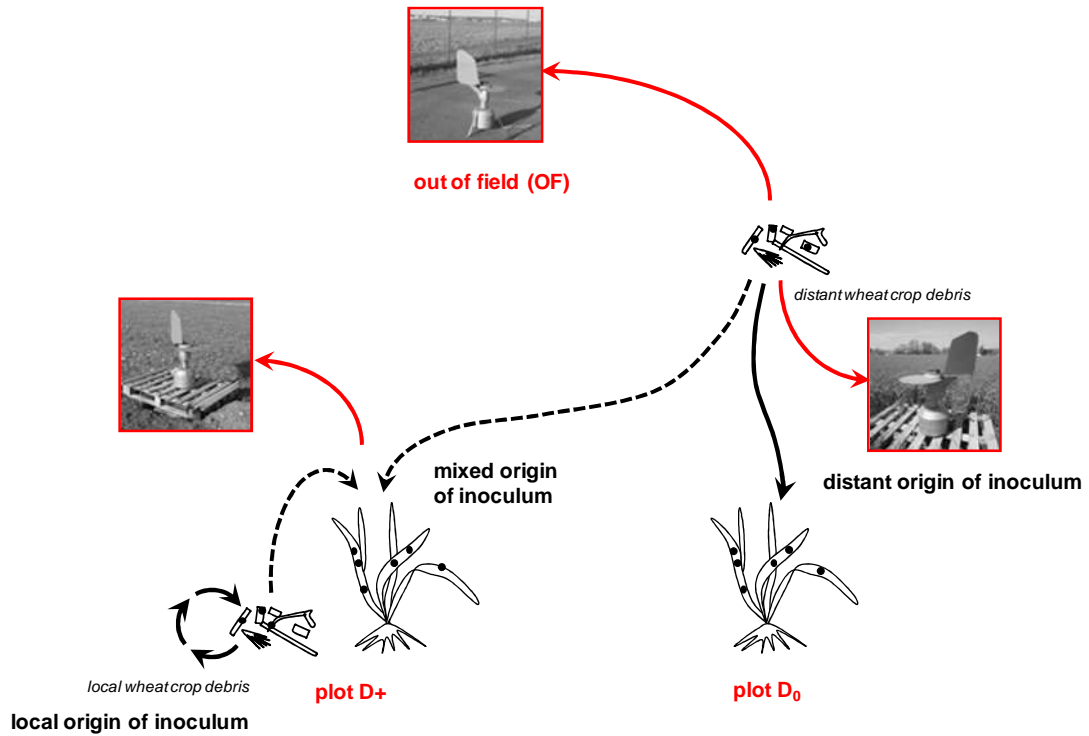


Figure 1 - Schematic representation of the origin of the *Zymoseptoria tritici* ascospores collected by the three spore traps according to their location. The D₀ and OF spore traps were expected to collect mainly distant primary inoculum (ascospores produced on distant wheat crops debris). The D+ spore trap was expected to collect both distant and local primary inoculum (ascospores produced on local wheat debris left on the soil of the D+ plot), in an unknown proportion.

Airborne ascospore trapping

Three 7-day recording volumetric spore traps (Burkard® Manufacturing Co., UK) were used to catch airborne *Z. tritici* ascospores during the first part of the epidemics (autumn and winter) in the two seasons. The traps were operated in the center of plot D+, in the center of plot D₀, and out of field (OF) 500 m apart from D+ and D₀ in a relatively windy location, respectively (Figure 1). The D₀ and OF spore traps were intended to collect mainly ascospores produced on distant wheat crops debris, while the D+ spore trap was intended to collect ascospores produced either on distant wheat crops debris or on local wheat debris left on the soil of the D+ plot. The orifice of the traps was located 0.7 m above the soil surface, so that only wind-dispersed ascospores were collected, pycnidiospores being not expected to be splashed-dispersed to that height (Shaw, 1987). The spore traps were ran continuously at the manufacturer's throughput (10 L.min⁻¹ / 14.3 m³ per day) over two periods, from 10 November 2011 to 11 March 2012 and from 9 October 2012 to 3 April 2013. Ascospores were caught on a Melinex® impacting tape coated with a thin layer of petroleum jelly. For each 7 day sampling period, the impacting tape was cut in seven 1-day sections (48 mm × 15 mm) using a Burkard® Perspex cutting block. Each section was enclosed in a box in order to prevent cross-contact between tapes. The boxes were kept at -80°C for several months until DNA extraction.

Quantification of ascospore DNA

For the sake of comparison between the three trapping locations, the tapes of the three spore traps operated during a same 7-day period were extracted concomitantly. Each 1-day tape section was placed in a Fast-Prep tube (2 mL) containing 80 µl of AP1 buffer (Qiagen Plant Mini Kit DNA, France) and 130 mg of glass beads (4.5 mm in diameter; Deutscher, France). DNA was extracted by series of 21 tubes. For each series, the positive control consisted in a suspension of pycnidiospores of *Z. tritici* (5000 pycnidiospores/50 µL) in 70% ethanol. All the positive controls came from a single pycnidiospore suspension prepared in January 2012 from infected wheat leaves and aliquoted in several tubes subsequently kept at -20°C. In order to detect contamination events during DNA extraction, a negative control containing sterile water was also used. The tubes were shaken in a Fast Prep machine (3 × 1 min at 6 M.s⁻¹) to disrupt and remove the ascospores from the tape. The Plant Mini Kit (Qiagen) was adapted for the material with slight modifications. 350 µL of AP1 buffer and 4 µL RNase A

(Qiagen) were added in each tube, before 5 min heating at 65°C. 6 µL of proteinase K (20 mg.mL⁻¹) was then added and tubes were heated again 10 min at 65°C. After centrifugation for 1 min at 20000 g, the supernatant was transferred to a new tube. The next steps of the DNA extraction followed Qiagen standard protocol. Extracted DNA was kept at -20°C.

Fungal DNA was quantified using the quantitative PCR assay developed for *Z. tritici* (Duvivier et al., 2013). The specific set of primers included a forward primer (5'-ATTGGCGAGAGGGATGAAGG-3'), a reverse primer (5'-TTCGTGTCCCAGTGCGTGTA-3'), both giving an amplification product of 101 pb, and a Taqman fluorogenic probe (5'-ACGACTCGCGGCTTTCACCCAACG-3'). The probe was labelled with a FAM fluorescent reporter dye and a BHQ-1 quencher. The quantification reaction was performed with the CFX96 Real time System C1000 Thermal Cycler (BIORAD, USA), using hard shell PCR 96-well WHT/CLR plates. The mix reaction was composed of reverse and forward primers at 500 nM per reaction, the probe at 500 nM per reaction in a final volume of 25 µL, with 5µL of DNA introduced per well. All samples (standard DNA, ascospore DNA from tapes, positive and negative controls) were analyzed with three replicates. The melting program was 95°C for 10 min and (95°C for 15 s, 60°C for 20 s, 72°C for 40 s) repeated for 40 cycles.

The concentration of DNA in the unknown samples was calculated by comparing threshold cycle (Ct) values of the samples with known standard quantities of *Z. tritici* genomic DNA, using a fivefold serial dilution from 0.5 ng to 5.10⁻⁵ ng per well. Ct values were plotted against the log of the initial concentration of *Z. tritici* genomic DNA to produce a standard curve. The validity of the standard curve of the quantification method was tested using a linear regression model coupled with an ANOVA analysis and a Fisher test.

The specificity of the quantification method (Duvivier et al., 2013) was confirmed by including DNA extracts from 11 *Z. tritici* isolates collected in the D+ plot (one of which was used as qPCR standard) and 8 *Septoria passerinii* isolates.

The limit of detection (LOD) of the qPCR assay was determined using a DNA standard curve consisting in a fivefold serial dilution from 0.5 to 5.10⁻⁵ ng of DNA. Dilutions 1, 2 and 3 (0.5, 0.05 and 0.005 ng of DNA) were done in triplicates whereas dilutions 4 and 5 (5.10⁻⁴ and 5.10⁻⁵ ng of DNA) were repeated 30 times. The LOD of a qPCR assay is usually considered to be reached when 90% of the replicates (here, 27/30) are detected (AFNOR,

2006). Here 100% of the replicates were detected for a given dilution D and less than 90% were detected for the dilution D/10. Accordingly, the theoretical LOD of the qPCR assay was between those two Ct values but could not be determined precisely. Consequently, we defined and used two LODs. The upper limit of detection (LOD_{up}) corresponded to the mean Ct value for dilution D and the lower limit of detection (LOD_{low}) corresponded to the mean Ct value for dilution D/10.

The limit of quantification (LOQ) of the qPCR assay was calculated using standard curves of all the plates considered as usable (see Results section). To this end, a Student's t test was used (AFNOR, 2006).

Each sample (1-day tape section) was assigned to a class corresponding to the position of the three Ct value of the sample regarding the limits LOQ, LOD_{up} and LOD_{low} . Samples with three replicated Ct values under LOQ were assigned to class I; ascospores collected in these samples were considered as "truly quantifiable". Samples with three replicated Ct values ranging from LOQ to LOD_{up} were assigned to class II; ascospores collected in these samples were considered as "quantifiable". Samples with three replicated Ct values ranging from LOD_{up} to LOD_{low} were assigned to class III; ascospores collected in these samples were considered as "detectable but not quantifiable". Samples with three replicated Ct values higher than LOD_{low} or with one or two undetermined Ct values were assigned to class IV; ascospores collected in these samples were considered as "not quantifiable either detectable". Samples with three undetermined Ct values were assigned to class V; these samples were considered as "ascospore-free". Samples were then assigned to one of three time periods (P1, P2, P3), specific for each year, according to the collection date. The limit between P1 and P2 in the first season and the limit between P2 and P3 in the second season were defined by the peak in the quantity of *Z. tritici* DNA. The limit was established by fitting a quadratic equation to the Ct values of all samples ("quantifiable", "detectable", and "not detectable") collected during the year. The limit between P2 and P3 for the first season, and between P1 and P2 for the second season, was fixed so that the same number of samples was assigned in each respective period. For the first season, P1 lasted from 10 November 2011 to 2 January 2012, P2 lasted from 3 January 2012 to 16 February 2012, and P3 lasted from 17 February 2012 to 11 March 2012. For the second season, P1 lasted from 9 October 2012 to 15 December 2012, P2 lasted from 16 December 2012 to 1 February 2013, and P3 lasted from 2 February 2013 to 3 April 2013.

Statistical analyses

The number of samples assigned to each class (I to V) and the quantity of DNA obtained by qPCR for a given sample were hereafter considered as explained variables. The time period (P1, P2, P3) and the spore trap location (D+, D₀, OF) were considered as explanatory variables. Firstly, the effect of spore trap location on the number of samples assigned to each class was investigated for the two seasons. A non parametric Kruskal-Wallis test was used to determine if there were significant differences in the number of samples assigned to each class between all spore traps or between two spore traps. Secondly, the effects of spore trap location and of the time period on the quantity of DNA were investigated, only for the samples assigned to classes I, II and V (for samples in which *Z. tritici* DNA was considered as “quantifiable” or “detectable”) in a factorial analysis of variance for the two seasons. The variance was partitioned into sources attributable to period (P), and spore trap location (T) according to the model:

$$Y_{ipt} = M + P + T + P \times T + \epsilon_{ipt}$$

where Y_{ipt} is the quantity of DNA of sample i , collected in period p , in spore trap t , and ϵ_{ipt} the error term.

For each of the two seasons, the means of quantity of DNA of each sample were then compared between periods and between spore traps with a Student-Newman-Keuls test. Statistical analyses were performed with the S-PLUS 6.3 software (Lucent Technologies, Inc., USA).

Estimation of the airborne ascospore concentration

For the sake of comparison with the literature, airborne ascospore concentration was derived from the quantity of DNA obtained with the qPCR assay. A standard curve using *Z. tritici* pycnidiospore suspensions at different concentrations was established. From a unique pycnidiospore suspension, three batches of dilutions were prepared separately. Seven dilutions were performed for each batch: 5000, 1250, 500, 250, 125, 50 and 25 spores in 50 μ L of 70% ethanol. Pycnidiospores were then disrupted and DNA extracted and quantified as described above. Pycnidiospores being actually made of four to eight cells (Shaner, 2010), we assumed they are made of six cells in average (Duvivier et al., 2013); ascospores are

bicellular. The relationship between the approximate amount of ascospores (y) and the total quantity of DNA (x) was established by using a linear equation. The airborne ascospore concentration ($\text{m}^{-3}\cdot\text{day}^{-1}$) was then obtained by dividing the estimated amount of ascospores by the volume of air sucked by the spore trap ($14.3 \text{ m}^3 \text{ day}^{-1}$).

Results

Disease assessment

No difference in plant development was observed between D+ and D₀ plots. At each sampling date, there was no difference in the biomass (g) of plants sampled in the two plots (data not shown). Accordingly, differences in epidemic development between the two plots could not be due to differences in the development of the plant stand.

The earliness of the *Septoria tritici* blotch epidemic was quite similar for the two seasons in plot D₀ (Table 2). The first lesions were detected on 1 December (430°C-days post sowing) and 29 November (390°C-days post sowing) during the first and second season, respectively. Six weeks later (on 9 January 2011 at 706°C-days post sowing and on 7 January 2012 at 625°C-days post sowing, respectively), the uppermost diseased leaf layers were leaf L5 of the main stem and leaf L1 of the first tiller in the first season, and leaf L3 of the main stem in the second season. This small difference in disease development was due to lower temperatures in December 2011 than in December 2012. Similarly, differences in disease development were low between the two seasons in plot D+ (data not shown).

In the first season, the epidemic developed earlier in plot D+ than D₀. Until mid-December (500°C-days post sowing), there was no difference between the two plots either in disease severity (Figure 2a) or incidence (Figure 2b), as assessed on leaf L1 and L2 of the main stem. Later on, disease severity and incidence were consistently higher in D+ than in D₀. The difference in disease severity peaked (20%) on 9 January 2011 (706°C-days post sowing), both on leaves L1 and L2. The difference in disease incidence peaked (more than

Table 2 - Wheat leaf layers infected by *Zymoseptoria tritici* but still partially green on main stem (MS) and first tiller (T1) at each sampling date in plot D₀ during seasons 2011-2012 and 2012-2013. For example, '2-6' means that layer 1 is completely senescent and that layers 2 to 6 have at least one infected leaf (neither completely senescent nor completely healthy) among the 3 × 25 plants.

	date	dd ^a	MS	T1
First season (2011-2012)				
sowing date	15/10/2011	0		
not1	16/11/2011	311		
not2	22/11/2011	358		
not3	01/12/2011	430	0-1	
not4	08/12/2011	488	1-2	
not5	20/12/2011	555	1-3	
not6	09/01/2012	706	1-5	1
not7	31/01/2012	815	1-6	1-3
not8	23/02/2012	865	2-6	1-3
not9	28/03/2012	1174	3-7	2-5
not10	24/04/2012	1390	5-8	4-7
not11	30/05/2012	1891	8-11	6-10
not12	21/06/2012	2224	10-11	9-10
Second season (2012-2013)				
sowing date	15/10/2012	0		
not1	20/11/2012	320		
not2	29/11/2012	390	0-1	
not3	11/12/2012	420	1-2	
not4	17/12/2012	453	1-3	
not5	07/01/2013	625	1-3	
not6	06/02/2013	727	2-5	1-2
not7	20/03/2013	861	4-6	1-4
not8	25/04/2013	1147	4-7	2-4
not9	22/05/2013	1446	5-8	2-5
not10	01/07/2013	2022	7-11	4-8

^a degree days post sowing, base 0°C

30%) on 8 December 2011 (488°C-days post sowing) on leaf L1 and on 20 December 2011 (555°C-days post sowing) on leaf L2.

In the second season, no clear trend in the earliness of the epidemic could be observed. There was no difference between plots D+ and D₀ in disease severity (Figure 2c) on leaf L1 and L2, except on 7 January 2013 (625°C-days post sowing), when severity on L2 was 20% lower in D+ than D₀. There was no difference either in disease incidence (Figure 2d) on leaves L1 and L2, except on 11 December (420°C-days post sowing; Figure 2d), when severity on L1 was 20% lower in D+ than D₀ while severity on L2 was 20% higher in D+ than D₀.

Quantification of ascospores on wheat debris

In the first season, no ascospore was collected on Petri dishes (PDA) from the wheat debris. In the second season, the production of ascospores during the early epidemic stage in plot D+ (Figure 3) was estimated to peak (15,000 ascospores.m⁻²) in October 2012, taking into account the mean debris surface density in the plot (470 g.m²), then decreased, and eventually stopped in March 2013.

Validation of the qPCR analysis

The published performance of the qPCR assay (Duvivier et al., 2013) was confirmed in our experimental conditions. The genomic DNA (gDNA) standard curve, established using the 29 plates (see below) considered as valid, had the following regression equation: $Ct = -3.374 \log(x) + 24.766$ ($R^2 = 0.992$), where x is the quantity of DNA to be determined. The efficiency of the quantification method was estimated at 97.9%. As expected, no amplification was obtained using gDNA of eight different isolates of the barley pathogen *S. passerinii* (data not shown). On the opposite, the gDNA of the 11 *Z. tritici* isolates was amplified.

For the 4th dilution (5.10⁻⁴ ng DNA) of the DNA standard curve used to establish the limits of detection of the qPCR assay, 100% of positive signals were obtained, with a mean Ct value of 36.41, while only 67% of positive signals were obtained for the 5th dilution (5.10⁻⁵ ng DNA) with a mean Ct value of 37.84. Consequently, the Ct values 36.41 (about 19±6 ascospores.m⁻³.day⁻¹) and 37.84 (about 13±4 ascospores.m⁻³.day⁻¹) were considered as the

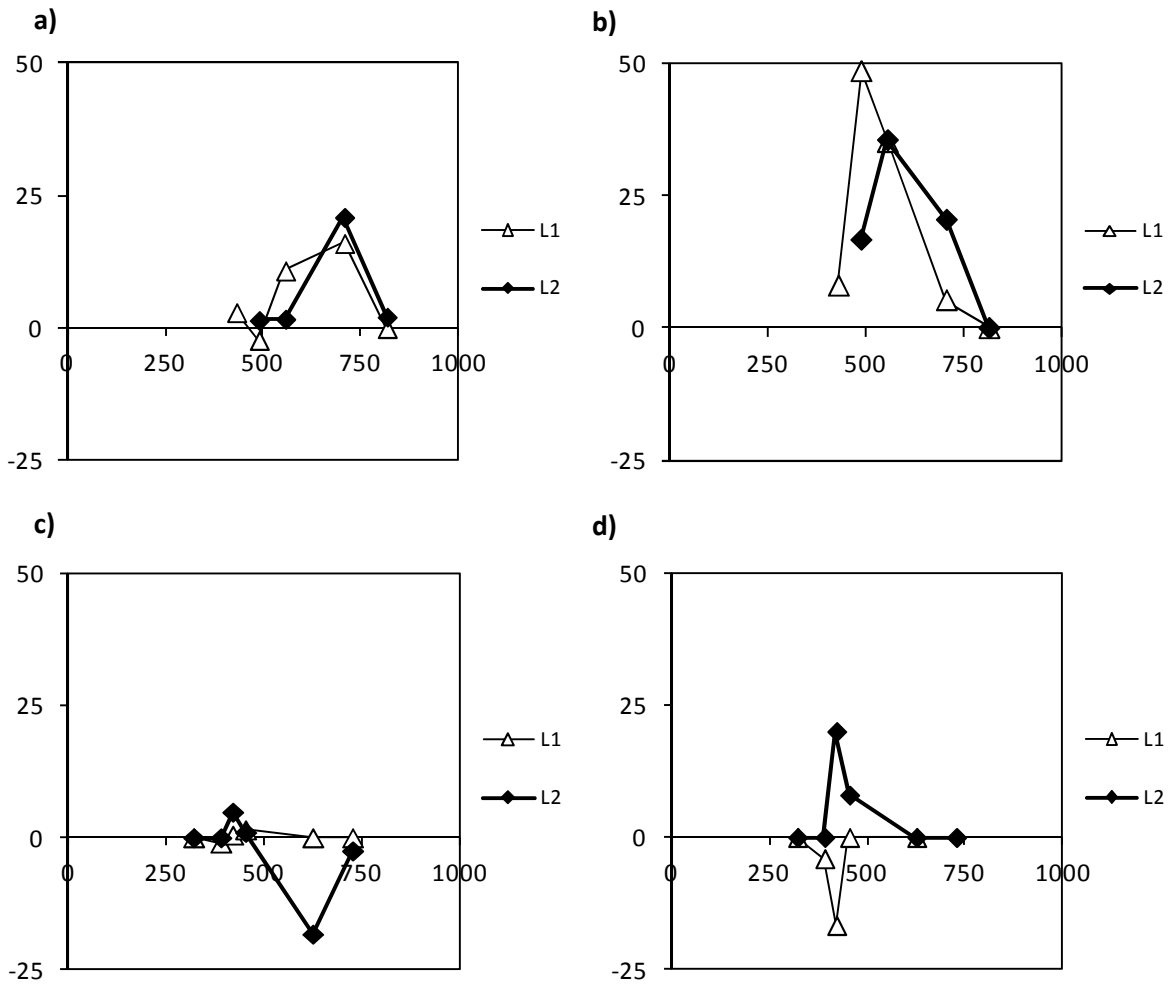


Figure 2 - Difference (%) in Septoria tritici blotch severity (a, c) and incidence (b, d) between D+ and D₀ plots for the first (2011-2012; a, b) and second (2012-2013; c, d) seasons. Disease severity and incidence were assessed on the first (\triangle) and second leaf (\blacklozenge) of the main tiller.

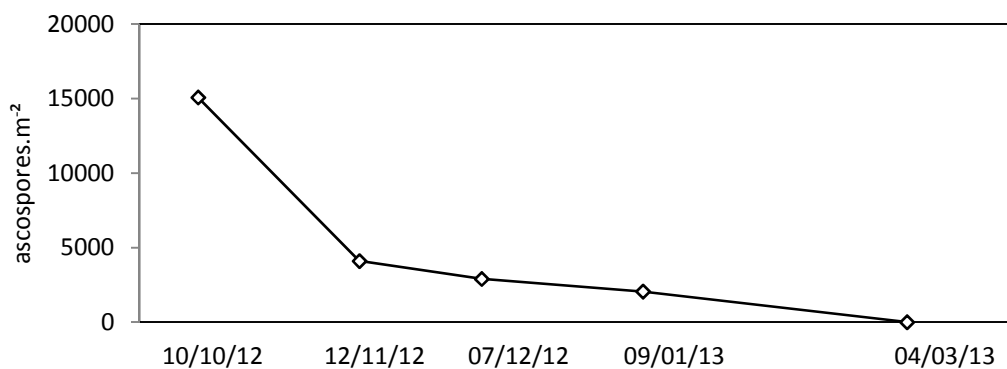


Figure 3 - Production of *Zymoseptoria tritici* ascospores on wheat debris collected in D+ plot during the early stages of the epidemic (season 2012-2013). The amount of ascospores was estimated by counting *Z. tritici* colonies resulting from ascospore ejection on Petri dishes (PDA). Mean debris density in the plot was 470 g.m⁻².

upper limit of detection (LOD_{up}) and the lower limit of detection (LOD_{low}), respectively. The limit of quantification (LOQ) was established at the Ct value 32.94 (about 121 ± 40 ascospores. m^{-3} .day $^{-1}$). Although the theoretical LOQ ranged between a Ct of 36.41 (4th dilution, Student's t = 1.012) and a Ct of 32.94 (3rd dilution, Student's t = 0.369).

The qPCR plates were validated against both positive and negative controls. When the mean Ct values of the three replicates of the positive control ranged between 29 and 31 (absence of false negative), the plate was declared valid. When the Ct value of at least one of the three replicates of the negative control was less than 38 (false positive), the plate was declared contaminated and non valid, and so excluded from the analysis. The application of these two criteria to the 40 plates run during the whole experiment left 29 plates considered as valid and subsequently used for the quantitative analysis. The remaining 11 plates (7 for the first season and 4 for the second season) were excluded.

The relationship between the amount of ascospores (y) and the quantity of DNA (x) was described by the linear equation $y = 7438.6 x + 41.2$ (Table 3). This equation gives an estimate of an airborne ascospores concentration (in ascospores. m^{-3} .day $^{-1}$) in relation to a quantity of DNA (ng).

Spatio-temporal variations in the amount of ascospore DNA

The qPCR assay was used to analyze 160 samples (1-day tape sections) in the first season and 427 samples in the second season (Figure 4). In the first season, 15.7% of the samples were considered as quantified (classes I and II), only two samples (1.3%) being assigned to class I (Table 4). Slightly more than half of the samples were considered as neither quantifiable nor detectable (class IV). Only one sample (0.6%) was ascospore-free. In the second season, 67.7% of the samples were considered as quantified, 53 of them (12.4%) being assigned to class I. Only 7.7% of the samples were considered as neither quantifiable nor detectable. Only one sample (0.2%) was ascospore-free. The class distribution of the samples was significantly different between the two seasons (Kruskal-Wallis test, $H = 155.6$, $P < 0.001$).

The mean quantity of DNA of the samples considered as quantified (classes I, II and V) was 0.017 ng for the first season (26 samples) and 0.063 ng for the second season (290 samples; Table 3). In average, the amount of ascospore DNA was nearly four times higher in the second than in the first season. When considering the time periods of trapping, most

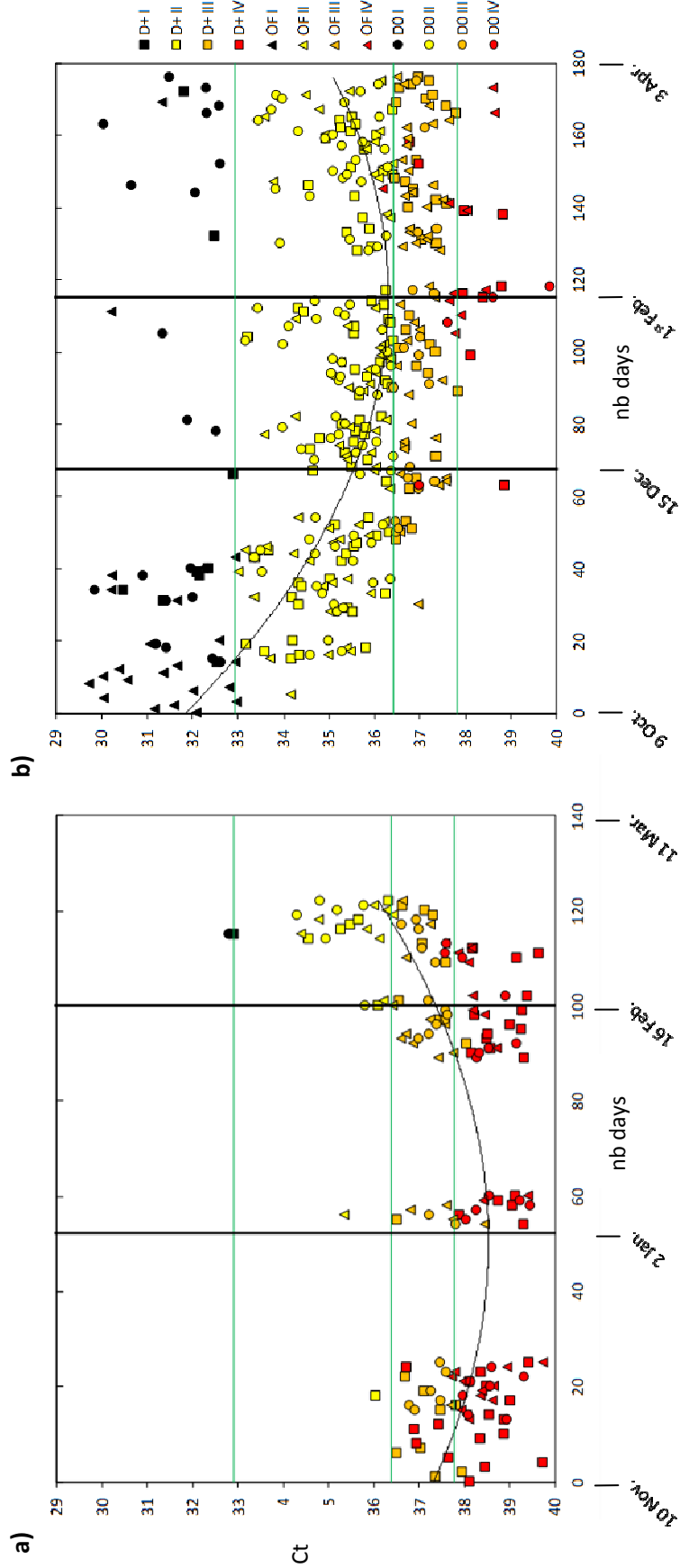


Figure 4 - Ct values for all samples (1-day tape sections) analyzed by qPCR for the first (2011-2012; a) and second (2012-2013; b) seasons. Each Ct value was calculated using three independent qPCR replicates. Samples were obtained from three different spore traps (O: D₀; Δ: D₊; □: D₊) and assigned to four classes (black, class I; yellow, class II; orange, class III samples; red: class IV). The limit of quantification (LOQ) and the upper and lower limits of detection (LOD_{up} and LOD_{low}) are indicated by horizontal green lines. The sampling period was split into three periods (P1, P2, P3), as defined in the main text. A quadratic curve ($y = a.x^2 + b$) was fit to the complete data sets.

Table 3 - Quantification of *Zyloseptoria tritici* pycnidiospore DNA by qPCR assay. Mean Ct values and mean DNA quantities were calculated using three independent qPCR replicates. A linear model ($y = 7438.6 x + 41.3$; $R=0.999$), fitted to the amount of pycnidiospores deposited on Melinex® tapes (y) and the total quantity of DNA (x), gives an estimate of the amount of pycnidiospores in relation to the quantity of DNA (ng).

Amount of deposited pycnidiospores (y)	Mean Ct value	Standard deviation	DNA quantity (ng)	Quantified amount of pycnidiospores ^a	Estimate amount of ascospores ^b	Airborne concentration (ascospores.m ⁻³ .day ⁻¹)	Sample status
5000	29.93	0.08	0.669	5022	15066	1054	class I
1250	31.95	0.33	0.147	1139	3417	239	class I
500	33.27	0.69	0.061	497	1491	104	class II
250	33.86	0.16	0.033	290	870	61	class II
125	35.41	0.59	0.011	127	381	27	class II
50	37.32	0.41	< 0.005	61	183	13	class III
25	37.31	0.42	< 0.005	63	189	13	class III

^a calculated using the equation of the linear model.

^b calculated by multiplying the estimated amount of pycnidiospores by three, taking into account the number of cells per type of spore (in average, six nuclei for a pycnidiospore and two nuclei for an ascospore; Shaner, 2010).

^c calculated by dividing the estimated amount of ascospores by 14.3 (see Materials & Methods section).

Table 4 - Number of samples assigned to each class (I, II, III, IV, V) for three spore traps and two seasons. A sample is a 1-day tape section.

First season (2011-2012)

Class	Quantified	Quantified	Detected	Not detected	Absent	Total
	I	II	III	IV	V	
Trap location						
D+	1	7	17	38	0	63
OF	0	10	16	22	0	48
D ₀	1	6	20	21	1	49
Total	2	23	53	81	1	160
Percentage per class	1.3	14.4	33.1	50.6	0.6	

Second season (2012-2013)

Class	Quantified	Quantified	Detected	Not detected	Absent	Total
	I	II	III	IV	V	
Trap location						
D+	9	79	39	13	0	140
OF	23	72	44	14	1	154
D ₀	21	85	21	6	0	133
Total	53	236	104	33	1	427
Percentage per class	12.4	55.3	24.4	7.7	0.2	

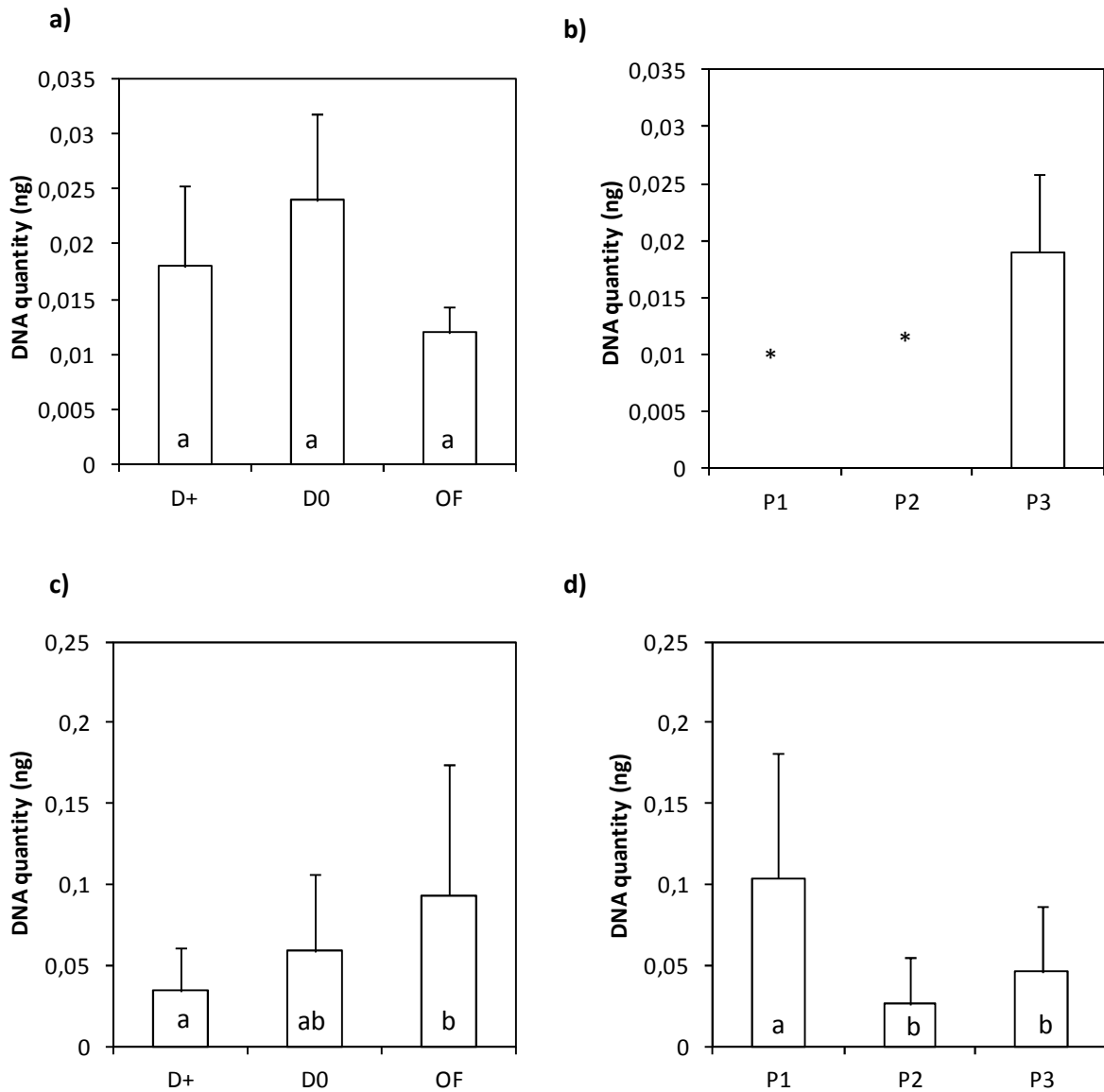


Figure 5 - Quantities (ng) of *Zymoseptoria tritici* DNA for samples belonging to classes I, II and V, according to the spore trap location (D+, D₀ and OF) and the time period (P1, P2 and P3) for the first (2011-2012; a, b) and the second season (2012-2013; c, d). Significant differences revealed by the Student-Newman-Keuls test ($P < 0.05$) are indicated by different letters. Note the difference in the scale of the vertical axis between the two years. In panel 3b, * corresponds to a single sample.

Table 5 - Effect of spore trap and trapping period on the amount of *Zymoseptoria tritici* DNA during the second season (2012-2013). Only samples assigned to classes for which DNA was considered as quantifiable or detectable (I, II and V) were kept.

	df	Mean Square	F	P
Trap	2	0.08	6.58	0.002
Period	2	0.15	12.69	< 0.001
Trap × Period	4	0.03	2.41	0.049
Residual	281	0.01		

Table 6 - Comparison of class distribution of samples (I, II, III, IV, V) between spore trap locations (D+, D₀ and OF) for two seasons.

First season (2011-2012)

	df	n ^a	H ^b	P
All traps	2	160	2.85	0.241
D+ vs. OF	1	111	2.41	0.121
D+ vs. D ₀	1	112	1.67	0.197
D ₀ vs. OF	1	97	0.09	0.761

Second season (2012-2013)

	df	n ^a	H ^b	P
All traps	2	427	12.54	0.002
D+ vs. OF	1	294	0,44	0.504
D+ vs. D ₀	1	273	12.63	< 0.001
D ₀ vs. OF	1	287	6.76	0.009

^a number of analyzed samples.

^b Kruskal-Wallis statistic.

quantifiable samples of the first season (23 out of 35) were obtained during period P3, while only one quantifiable sample was obtained in each of the P1 and P2 periods. In the second season, the quantity of DNA in the samples significantly differed between the three time periods ($P < 0.001$; Table 5). The quantity of DNA was significantly higher in period P1 (0.105 ng) than in periods P2 and P3 (0.026 ng and 0.046 ng, respectively; Figure 5d); the difference in the quantity of DNA between periods P2 and P3 was, however, not significant.

In the first season, spore trap location had no significant effect on the number of samples assigned to each class (Table 6). No significant difference was found either in the pairwise comparison of the spore traps. In the second season, the effect of spore trap location was significant; the class distribution of samples in trap D₀ was significantly different from the other traps. However, fewer samples were quantifiable in D₊.

In the first season, the quantity of DNA of the samples considered as quantified did not significantly differ between spore traps (data not shown). In the second season, significant differences were found (Table 5). Pairwise comparison of spore traps showed a significantly higher quantity of DNA in OF than in D₊ trap (0.093 ng and 0.035 ng, respectively; Figure 5c), while the quantity of DNA on D₀ trap was not significantly different from either D₊ or OF trap.

Discussion

In this two-year study performed at a small spatiotemporal scale, we concomitantly surveyed the onset of disease epidemics and the variation in the airborne concentration of the spores purportedly acting as the initial inoculum. The assessment of initial, low levels of disease severity and incidence was performed as described earlier (Suffert & Sache, 2011). For the sake of reliability and comparison with other studies, we used a Burkard spore trap, which is the standard device for the capture of airborne micro-organisms (Jackson & Bayliss, 2011). The main technical challenge in this study was the detection and quantification of small amounts of airborne spores. Reports of detection and quantification of one or two spores (Parker et al., 2014, Carisse et al., 2009; Rogers et al., 2009) using a serial dilution of DNA of a spore suspension are questionable, because of loss of DNA during extraction steps. To validate our qPCR assay, we assessed both its limit of detection (LOD) and its limit of quantification (LOQ). Those two limits are often not explicitly stated in the literature (e.g.,

Haegi et al., 2013; Parker et al., 2014). The ‘detection threshold for quantification’ reported for *Z. tritici* ascospores (Fraaije et al., 2005) is equally ambiguous.

LODs and LOQ were derived from standard curves established with gDNA extracted from *Z. tritici* pycnidiospores. The use of gDNA (Lees et al., 2012) rather than plasmid cDNA (e.g., Duvivier et al., 2013), allowed the PCR assay to be closely as efficient as it would have been with ascospore DNA. The assignment of samples to the classes established using LOD_{up}, LOD_{low} and LOQ allowed to exploit the full data set and to perform statistical analyses. The definition of ‘detectable samples’ allowed an overall comparison between traps, while ‘quantifiable samples’ were used to compare daily patterns of ascospore releases.

The main drawback of our method is the lack of direct comparisons of the number of spores actually trapped and the quantity of DNA quantified using the assay. The link between the amount of ascospores and genomic DNA amount was indirectly obtained, using suspensions of *Z. tritici* pycnidiospores. The limits of detection eventually calculated were in accordance with thresholds established on others pathogens (e.g., Lees et al., 2012; Schweigkofler et al., 2004; Klosterman et al., 2014).

The effect of the year on the airborne ascospore concentration was consistent with the significant difference in ascospore production from contaminated debris. Such a difference is likely to occur also in other fields with wheat debris in the Grignon basin, and could be related to the severity of disease on the previous year (Cowger et al., 2002): the more severe are epidemics on a season, the more frequent is the sexual reproduction on debris and the higher will be the amount of ascospores released from these debris at the beginning of the next season. Severity was high in 2011-2012 (lesions covered 15-20% of the flag leaf area at mid-June 2012), while it had been very low in 2010-2011 (lesions were absent from flag leaf at mid-June 2011); sexual reproduction was, most probably, more frequent during the late epidemic stages at autumn 2012, as evidenced by the high amounts of ascospores either collected from debris (Figure 3) or caught by the spore traps (Figure 4).

The amount of ascospores responsible of the first infections occurring immediately after seedling emergence (trapped in period P1) was about ten-fold higher in late autumn 2012 than in late autumn 2011. Among the highest daily peaks of ascospores in that period, we identified 22 daily peaks in 2012-2013 with more than 230 ascospores.m⁻³.day⁻¹ (Ct > 32; Table 3; Figure 4b), from 10 October to 18 November, but none in 2011-2012. Such a

magnitude of airborne ascospore concentration is consistent with observations made in wheat crops in autumn: a maximum of 92 ascospores.m⁻³.day⁻¹ (Bathgate & Loughman, 2001), between 50 and 125 ascospores.m⁻³.day⁻¹ (Hunter et al., 1999), and between 100 and 350 ascospores.m⁻³.day⁻¹ (Duvivier et al., 2013). This confirmed the occurrence of peaks in ascospore production in autumn, concomitantly with wheat emergence.

Peaks of ascospore concentration were also recorded in the subsequent time periods in 2012-2013: one in December and two in January (period P2), four in March and one in April (period P3). Accordingly, ascospores were present in air almost all the autumn and winter periods as established in previous studies (Hunter et al., 1999; Bathgate & Loughman, 2001).

The local presence of infected wheat debris had no obvious effect on the amount of airborne *Z. tritici* ascospores. No difference was found between the three spore traps in season 2011-2012. This was not surprising since the wheat debris in D+ were not considered as a significant source of inoculum (ascospore production on these debris was undetectable). In season 2012-2013 the airborne ascospore concentration was not correlated with the presence of a local source of inoculum. Other factors may explain local differences in airborne ascospore concentration, such as topography and wind currents.

The local presence of infected wheat debris had no obvious effect either on the earliness of the epidemic, especially the year when inoculum pressure was high (2012-2013). The epidemic was earlier in the plot where wheat debris were present but less contaminated (D+), in the season when inoculum pressure was low (2011-2012). Conversely, no difference in earliness was found between the plot without debris (D₀) and the plot with infected debris (D+), in the season when debris were highly infected (2012-2013). In spite of the limited duration of the experiment (two years), these results suggest that the amount of airborne ascospores available over a wheat crop is not a limiting factor for the early stages of *Septoria tritici* blotch epidemics (Holmes & Colhoun, 1975; Shaw & Royle, 1989). In accordance with previous results (Suffert & Satche, 2011), we believe that the earliness of a *Septoria tritici* blotch epidemic is mostly determined by the sowing date: whatever its date, the airborne ascospore concentration is usually not limiting so that the first infection by the fungus occurs as soon as the first leaf emerges. This implies that management of primary inoculum at a local scale by removing wheat debris from nearest fields offers little help to *Septoria tritici* blotch control in Western Europe conditions.

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Chapitre III

Pathogénicité comparée de spores sexuées et asexuées de *Z. tritici*

Comparative pathogenicity of sexual and asexual spores of *Zymoseptoria tritici* (*Septoria tritici* blotch) on wheat leaves *

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Abstract

Zymoseptoria tritici ascospores and pycnidiospores are considered as the main forms of primary and secondary inoculum, respectively, in *Septoria tritici* blotch epidemics. We compared the pathogenicity of the two types of spores of the same genotypic origin through a two-staged inoculation procedure in controlled conditions. Ascospores collected from field sources were inoculated on adult wheat leaves and yielded 119 lesions; pycnidiospores collected from 12 lesions resulting from these ascospore contaminations were then inoculated. Lesion development was assessed during five weeks; latent period, lesion size, and pycnidium density were estimated for different isolates. Latent period was calculated as the maximum likelihood time elapsed between inoculation and either the appearance of the majority of the sporulating lesions (leaf scale) or the appearance of the first pycnidia (lesion scale). Latent period was significantly longer (~60 degree-days, i.e. 3-4 days) after contamination with ascospores than with pycnidiospores. No difference was established for lesion size and density of pycnidia. A comparison with other ascomycete fungi suggested that the difference in latent period may be related to the volume of spores and their ability to cause infection. Fungal growth before the appearance of lesions may be slower after inoculation with an ascospore than with a pycnidiospore. The mean latent period during the very beginning of epidemics, when first lesions are mainly caused by ascospores, may be longer than during spring, when secondary infections are caused by pycnidiospores. Disease models would be improved if such difference were considered.

Keywords

Ascospore, Gompertz function, latent period, *Mycosphaerella graminicola*, pycnidiospore, wheat.

Introduction

The ascomycete fungus *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*, anamorph *Septoria tritici*; Quaedvlieg *et al.*, 2011), causes Septoria tritici blotch, a foliar disease of wheat (*Triticum aestivum*) found in most wheat-growing areas worldwide. In Western Europe, the disease can induce up to 30-40 % crop loss when the upper leaves are severely contaminated (Eyal *et al.*, 1987). Disease epidemics are initiated by airborne sexual ascospores, mainly produced during the intercrop period (after harvest in summer and autumn) on contaminated wheat debris, which are considered as the main primary inoculum (Shaw & Royle, 1989; Hunter *et al.*, 1999; Suffert *et al.*, 2011). The disease is clonally propagated among wheat plants during the growing season by pycnidiospores, which are splash-dispersed over short distances and act as the main secondary inoculum. However, ascospores and pycnidiospores can be jointly involved into different stages of the epidemic development. In a wheat monoculture system, when contaminated debris is not completely buried, both pycnidiospores and ascospores can initiate the epidemic (Suffert *et al.*, 2011; Suffert & Satche, 2011). In spring, secondary infections can be induced by ascospores (Clinckemaillie *et al.*, 2010; Duvivier *et al.*, 2013) released either from old, still infectious debris located in distant plots or from infected wheat plants located in the same plot. Field reports of infection of the uppermost (F1) and lowermost green leaves (F3-F4) while the middle leaves (F2) remained healthy (C. Maumené, Arvalis-Institut du Végétal, Boigneville, personal communication) could be explained by such secondary infection by ascospores. Accordingly, specific stages of the epidemic development appear to be induced by one type of spore rather than the other, although the two types of spores are actually released. The preeminence of one type of spore has been explained by contrasted distances of dispersal (Shaw, 1987; Shaw & Royle, 1989) and spore amounts (Suffert & Satche, 2011), but rarely by differences in pathogenicity between sexual and asexual spores, as measured for example by the number of sporulating lesions they induce (Karolewski *et al.*, 2002; Trapero-Casas & Kaiser, 2007).

In fungi with alternating reproduction regime, the comparison of the pathogenicity of spore types is experimentally challenging. In *Z. tritici* the production and handling of large amounts of ascospores is cumbersome in artificial conditions (Kema, 1996). Conidia, which are asexual spores produced from yeast-like mycelium grown in liquid or solid culture media, were used to inoculate wheat plants (e.g., Cowger & Mundt, 2002; Leyva-Mir *et al.*, 2008;

Suffert *et al.*, 2013) rather than pycnidiospores produced on infected leaves. Whether conidia and pycnidiospores have the same pathogenicity has not been tested yet. Finally, the use of ascospores and pycnidiospores of the same genotype is required to perform a valid comparison, due to the high genetic diversity of *Z. tritici* populations (Linde *et al.*, 2002).

A first, main component of pathogenicity of *Z. tritici* is sporulation (Suffert *et al.*, 2013), assessed through the measurement of maximum sporulating area (e.g., Arraiano *et al.*, 2006), of pycnidium density (e.g., Simon & Cordo, 1997) and sporulation capacity (Eyal, 1971). A second main component of pathogenicity in *Z. tritici* is latent period (Suffert *et al.*, 2013), which reflects the pathogen's generation time. Latent period is usually defined as the time interval between infection and the onset of sporulation from that infection (Pariaud *et al.*, 2009), in the case of Septoria tritici blotch, the time elapsed between infection and the appearance of first pycnidia (Shearer & Zadoks, 1972). When a population of lesions rather than a single lesion is considered, as usual in experimental studies, the latent period is estimated, at the scale of a leaf, as the maximum likelihood time elapsed between inoculation and the appearance of the majority of the sporulating lesions (Shaw, 1990; Lovell *et al.*, 2004; Zearfoss *et al.*, 2011), or, at the scale of a lesion, as the maximum likelihood time elapsed between inoculation and the appearance of the first pycnidia (Armour *et al.*, 2004; Suffert *et al.*, 2013).

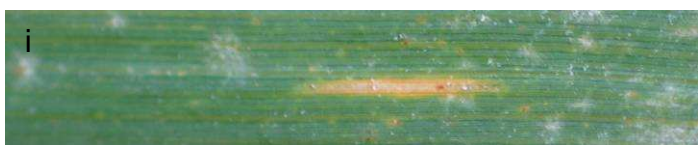
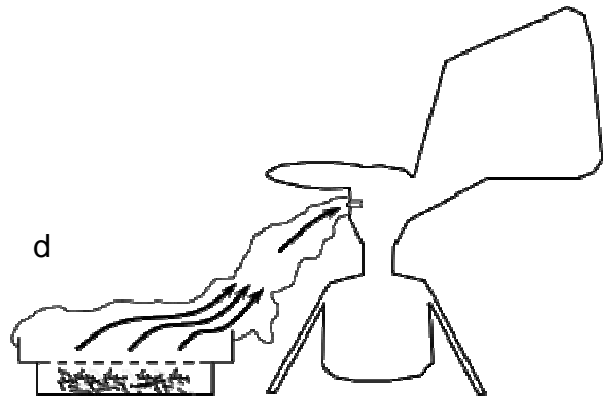
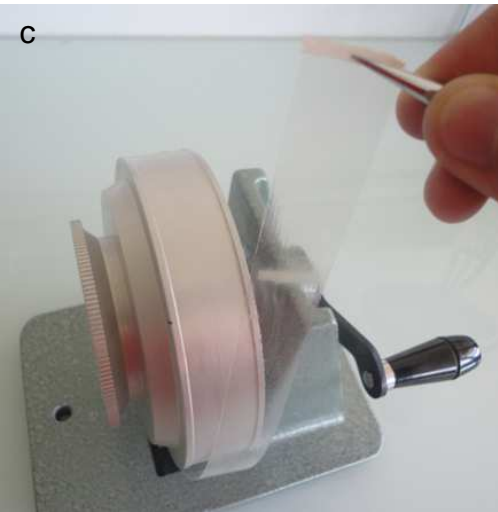
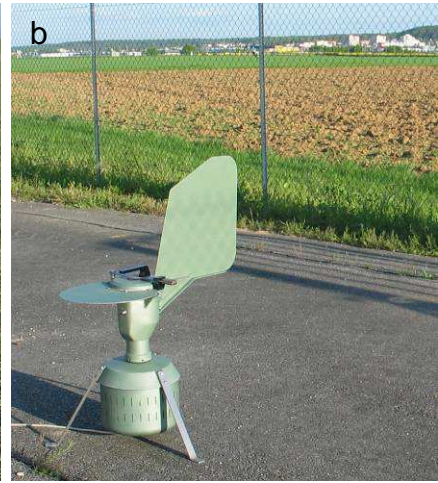
The objective of this study was to compare the pathogenicity of sexual and asexual spores of *Z. tritici* of similar genotypes, following infection of adult wheat leaves. Firstly, *Z. tritici* ascospores, collected from different inoculum sources, were inoculated on adult wheat leaves and lesion development was assessed. Secondly, pycnidiospores resulting from the ascospore inoculation (having so the same genotype as those ascospores) were collected at the end of the experiment and were directly inoculated on adult wheat plants at the same leaf stage. Differences in pathogenicity between the two spore types were compared by assessing the sporulation and latent period.

Material & Methods

Fungal material

Two plots were sown 300 m apart with wheat cv. Soissons (moderately susceptible to Septoria tritici blotch; rated 5 on a 1-10 scale of decreasing susceptibility, Arvalis Institut du

Figure 1 - Experimental design used to compare the pathogenicity of ascospores and pycnidiospores of *Zymoseptoria tritici*. (a) Burkard® spore trap placed in wheat monoculture plot (D+) with wheat debris (Grignon). (b) Burkard® spore trap placed out of field 300 m apart (Grignon). (c) Melinex® tape wound around the periphery of the rotating drum of the Burkard® spore trap. (d) Wheat debris placed in a bag above a Burkard® spore trap in order to compress the flow of ejected ascospores. (e) Inoculated leaves enclosed in a bag to promote infection. (f-i) *Septoria tritici* blotch lesions after inoculation with ascospore suspensions.



Végétal-CTPS) in autumn 2011 at the Grignon experimental station (France, 48°51'N, 1°58'E). Soil and weather characteristics, as well as cropping systems, have been described elsewhere (Suffert & Sache, 2011). The first plot (D+) had been grown by direct drilling and so contained wheat debris. The debris, hardly contaminated after a mild disease epidemic the previous season, constituted a weak source of ascospores (Suffert & Sache, 2011). The second plot (D₀), grown with oilseed rape the previous season, did not contain any wheat debris.

Airborne *Z. tritici* ascospores were caught in March and April 2012 using three 7-day recording volumetric Burkard® spore traps (Burkard Manufacturing Co., Rickmansworth, UK) placed 0.7 m above the soil surface in plot D₀, in plot D+ (Fig. 1a) and out of the field (Fig. 1b). The traps were operated at a flow rate of 10 L.min⁻¹ and the collecting surface of Melinex® tapes were covered, or not, with a thin layer of petroleum jelly (Vaseline®) before sampling (Fig. 1c).

Wheat debris were collected on 28 March 2012 from the plot D+ and from a wheat field (cv. Soissons) grown at the Versailles experimental station (France, 48°48'N, 2°04'E); at this location, disease was more severe the previous year than in Grignon (M. Leconte, personal communication). Debris were placed in laboratory conditions in two plastic grill boxes and sprayed with a small amount of water to induce the moisture change required to trigger the ejection of ascospores. Boxes were covered by a plastic bag connected to the trap aperture (Fig. 1d), in order to maximize the amount of collected ascospores. Two methods of trapping were used. Melinex® tapes covered, or not, with a thin layer of petroleum jelly (Vaseline®) and glass slides covered with petroleum jelly were placed in a 7-day and 24-hour volumetric Burkard® spore trap, respectively. The traps were operated at a flow rate of 10 L.min⁻¹.

All along the collection period, Melinex® tape and glass slides were kept for a few days at -20°C to limit ascospore decay or germination. Finally, 10 different supports corresponding to 10 ascospore trapping events were obtained during a 3-week period (Table 1).

Plant material

Two batches of wheat adult plants, cv. Soissons, were grown in two greenhouse compartments for subsequent inoculation. The two batches were sown in Jiffy peat pots on 7

December 2011 and 9 January 2012, respectively. Seedlings were vernalized for seven weeks at 8°C with a 10 h light period and a 14 h dark period. Afterwards, seedlings were transferred back to the greenhouse and left to acclimatize for one week. Plants were then transplanted into 1.1 L pots filled with 1 litre of commercial compost (Klasmann Peat Substrat 4; Klasmann, France) and fertilized with 5 g Osmocote Exact (16N 11P 11K 3MgO Te). Plants were also sprayed with Spiroxamine (Aquarelle SF at 2 ml. l⁻¹; Bayer Crop Science, France) against powdery mildew (*Blumeria graminis*). In addition, plants were fertilized twice with a commercial nutrient solution (1 l Hydrokani C2) diluted 1/100 and applied in the pot saucers five and seven weeks after the seedlings transplantation. During plant growth, natural daylight was supplemented with 400 W sodium lamps to obtain 9h of dark and 15h of light, the greenhouse compartment temperature being kept under 20°C during the light period and above 12°C during the dark period. Three stems per pot were kept. In each greenhouse compartment the air temperature was recorded every 15 minutes and the thermal time *t*, expressed in degree-days post inoculation (ddpi), was calculated starting from the inoculation dates by summing the daily mean air temperature using a 0°C base temperature.

Inoculation with ascospores

The day before the inoculation, 10 ascospore suspensions corresponding to the 10 aforementioned ascospore trapping events were prepared differently according to the type of support (Melinex® tape or glass slide) and to the presence of petroleum jelly or not. Melinex® tape were cut in several sections, each matching with a daily trapping. Each tape section was placed in a conical tube containing 10 mL of water and 130 mg of glass beads (4.5-mm in diameter, Deutscher, France). The tubes were manually shaken during 1 min. The tape sections covered with petroleum jelly were placed in a 65°C water bath during 5 min and centrifuged at 10000 rpm during 5 min to remove the ascospores from the tape. Glass slides covered with petroleum jelly were placed in a conical tube containing 10 mL of water then placed in a 65°C water bath during 5 min and centrifuged at 7000 rpm during 5 min. The contents of the tubes corresponding to the same trapping event (Table 1) were gathered and kept for a one day at 4°C to limit ascospore decay or germination.

Two drops of surfactant (Tween20®, Sigma, France) were added to the spore suspensions to ensure adequate coverage of the inoculated leaf surface. On 17 April 2012, each spore suspension was applied using a paintbrush along the adaxial face of the upper leaf

Table 1 - Origin of the 10 *Zymoseptoria tritici* inoculum sets used in the experiment.

Location	Origin of sampling	Support	Petroleum jelly	Collect duration	Period (2012)	Nb asc. ^a	Nb pyc. ^b	Isolates ^c
outside ^d	out of field (Grignon)	Melinex tape	no	7 days	28/03 to 03/04	14		
	plot D ₀ (Grignon)	Melinex tape	yes	14 days	13/03 to 26/03	12		
	plot D ₀ (Grignon)	Melinex tape	no	7 days	28/03 to 03/04	7		
	plot D+ (Grignon)	Melinex tape	yes	14 days	13/03 to 26/03	16		
	plot D+ (Grignon)	Melinex tape	no	14 days	06/04 to 12/04 and 28/03 to 03/04	8		
	plot D+ (Grignon)	Melinex tape	yes	14 days	13/03 to 26/03	16		
inside ^e	debris from plot D+ (Grignon)	Melinex tape	no	10 days	04/04 to 09/04 and 06/04 to 09/04	15	8	FS3307, FS3309, FS3344, FS3346, FS3356, FS3357, FS3385, FS3386
	debris from wheat plot (Versailles)	Glass slide	yes	2 days	04/04 to 06/04	12	4	FS3306, FS3314, FS3327, FS3359
	debris from wheat plot (Versailles)	Melinex tape	no	6 days	10/04 to 15/04	7		
	debris from wheat plot (Versailles)	Glass slide	yes	2 days	02/04 to 04/04	12		

^a total number of leaf lesions appeared after inoculation with the ascospore suspension ; ^b total number of leaf lesions selected for the subsequent inoculation with the pycnidiospore suspension ; ^c code of the 12 *Z. tritici* isolates which were inoculated both as ascospore and pycnidiospore type ; ^d location of the Burkard® spore trap (see Figure 1a, 1b) ; ^e plot without wheat debris (oilseed rape as previous crop) ; ^f plot with debris (wheat monoculture since 2007) ; ^g trapping device placed above debris (see Figure 1c, 1d).

(F1) of the two main tillers of five plants (10 leaves) of the first batch of plants. Each leaf was then enclosed for 72 h in a transparent bag (Fig. 1e) slightly wetted to maintain a high humidity and promote infection (Suffert *et al.*, 2013).

Inoculation with pycnidiospores

Twelve lesions were selected out of the 119 that had appeared after inoculation with ascospores (Fig. 1f-i) as a source of pycnidiospores (see Table 1 and Results section). The 12 lesions originated in ascospores trapped from debris of plot D+ either on Melinex® tape without petroleum jelly (8/12) or on glass slides (4/12), in order to ascertain the origin of the inoculum (local pathogen population) and so increase the reproducibility of the experiment. On 28 May 2012, after the last assessment of lesions caused by ascospore infection (see below), pycnidiospores were collected from each of the 12 selected lesions by placing the leaf in a 25 mm diameter tube containing 10 mL of water and softly sweeping the area covered by pycnidia with a paintbrush. Each pycnidiospore suspension was then immediately applied using a paintbrush along the adaxial face of the upper leaf (F1) of the main tiller of four plants (12 × 4 leaves) of the second batch. Each leaf was then enclosed for 72 h in a transparent bag, as performed previously for the inoculation with ascospores.

Assessment of pathogenicity components

Disease symptoms were assessed by the same assessor twice a week from 350 to 710 ddpi, from 11 May to 28 May 2012 for ascospore inoculation (7 assessments) and from 18 June to 4 July 2012 for pycnidiospore inoculation (8 assessments). The sporulating lesions, neither spatially aggregated nor coalescent, could be counted individually (maximum 3 lesions per leaf after ascospore inoculation; maximum 26 lesions per leaf after pycnidiospore inoculation). The length and width of each lesion were assessed visually using a ruler graduated in increments of 0.5 mm. The number of pycnidia born by each lesion was counted with a hand lens (magnification ×10).

The maximum size of a lesion ($SIZE_{max}$, mm^2) was calculated as the product of its length by its width.

A Gompertz function (Berger, 1981) was fitted to the number of pycnidia $PYC(t)$ recorded on each lesion (Suffert & Sache, 2013):

$$PYC(t) = PYC_{\max} \exp(-B_{PYC} e^{-r_{PYC} \cdot t}) \quad (\text{eq. 1})$$

where PYC_{\max} is the maximum number of pycnidia reached at the end of the assessment period, r_{PYC} is a rate parameter, B_{PYC} is a position parameter, and t is the time of observation expressed in degree-days post inoculation (ddpi). The density of pycnidia (PYC_{dens}) was then calculated as:

$$PYC_{\text{dens}} = \frac{PYC_{\max}}{SIZE_{\max}} \quad (\text{eq. 2})$$

The latent period was assessed using two complementary methods. Firstly, $LatPYC_5$, the time elapsed from inoculation to the appearance of 5% of the maximum number of pycnidia (PYC_{\max}), was calculated using eq. 1 for each lesion induced by each isolate, after inoculation either with ascospores or pycnidiospores. PYC_{\max} ranging from 20 to 80, $LatPYC_5$ was considered as a good indicator of the time elapsed between inoculation and the appearance of the 1-4 earliest pycnidia. Secondly, a Gompertz growth curve was fitted to the total number of sporulating lesions $LES(t)$ (i.e. lesions for which $PYC(t) \geq 0.05 \times PYC_{\max}$) assessed on several leaves:

$$LES(t) = LES_{\max} \exp(-B_{LES} e^{-r_{LES} \cdot t}) \quad (\text{eq. 3})$$

where LES_{\max} is the maximum number of lesions, r_{LES} is a rate parameter, and B_{LES} is a position parameter.

$LatLES_{37}$, the time elapsed from inoculation to $1/e \approx 36.8\%$ of LES_{\max} (inflection point of a Gompertz function), was calculated using eq. 3. This method could not be used after ascospore inoculation since each individual was unique and caused only one lesion.

Data analysis

$SIZE_{\max}$, PYC_{dens} and $LatPYC_5$ were estimated after ascospore inoculation for each of the 119 isolates, based on the assessment of a single lesion (no replication), and after pycnidiospore inoculation by mean values calculated from the assessment of all the lesions induced by each of the 12 selected isolates (Table 1).

For the ascospore inoculation, $LatLES_{37}$, calculated using the 119 lesions, was an average estimate of the latent period for 119 different isolates. For the pycnidiospore

inoculation, LatLES₃₇ was estimated firstly for a given isolate (among the 12 selected isolates), and secondly for the whole population using all the data corresponding to the 12 isolates.

The effect of the type of spore on the three pathogenicity components (SIZE_{max}, PYCdens, and LatPYC₅) was tested in an analysis of variance (ANOVA) (Table 2). For each component assessed on 12 isolates, the variance was partitioned into sources attributable to the type of spore (S), according to the model:

$$Y_{si} = M + S + \varepsilon \quad (\text{eq. 4})$$

where Y_{si} is the value of pathogenicity component Y of isolate i, for type of spore s, and ε the error term.

The mean of each pathogenicity component was compared between the type of spore as factor (Student-Newman-Keuls test).

The effects of petroleum jelly (with or without) and the origin of inoculum (Versailles, Grignon) on the latent period for the ascospore inoculation was tested in an analysis of variance (ANOVA) (Table 3). For LatPYC₅ assessed on 119 isolates, the variance was partitioned into sources attributable to the presence of petroleum jelly (J) and the origin (O), according to the model:

$$Y_{joi} = M + J + O + \varepsilon \quad (\text{eq. 5})$$

where Y_{joi} is the value of latent period of isolate i, for the presence or not of petroleum jelly j, for the origin o, and ε the error term.

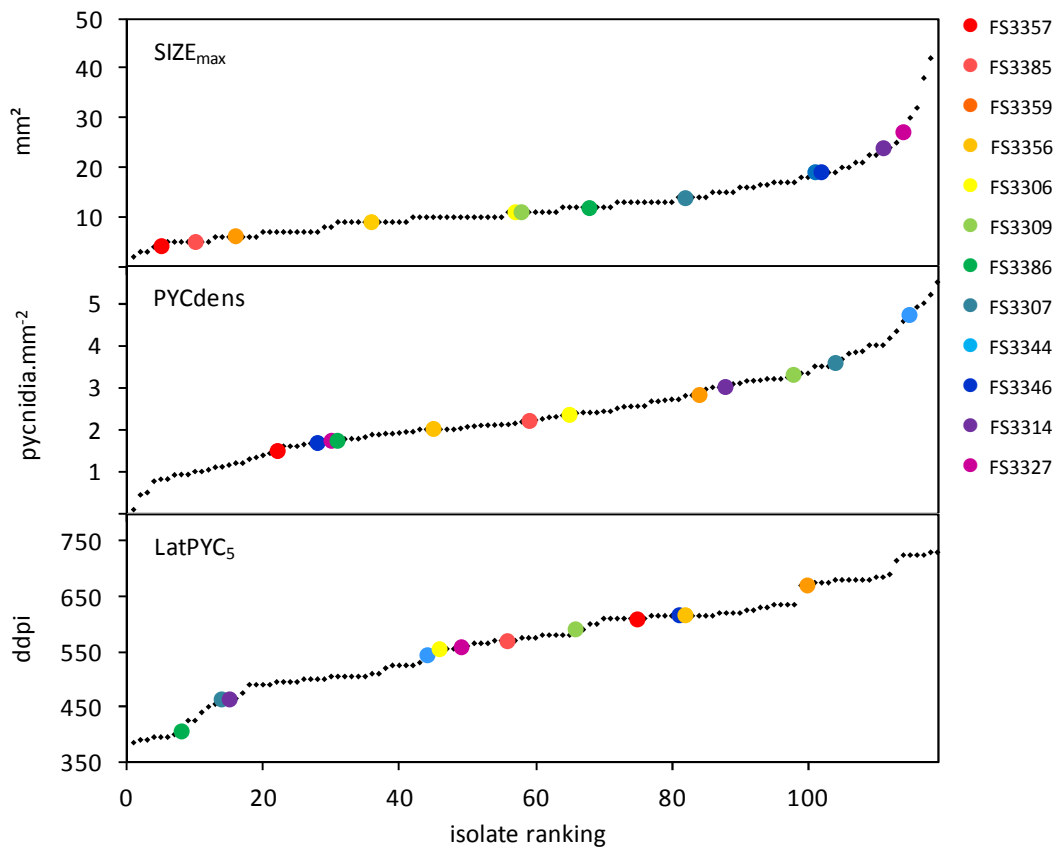


Figure 2 - Distribution of lesion size ($SIZE_{max}$), pycnidium density (PYCdens), and latent period (LatPYC₅) of the 12 *Zymoseptoria tritici* isolates (●) selected among 119 (◆) assessed after ascospore inoculation on adult wheat leaves.

Results

The inoculation of 100 wheat leaves with 10 ascospore suspensions yielded 119 lesions (Figure 1 f-i). One hundred lesions were induced by ascospores trapped from wheat debris at Grignon (against 19 from wheat debris at Versailles) and 68 were trapped using petroleum jelly (against 51 without) (Table 1). The inoculation of 12×4 wheat leaves with the 12 pycnidiospore suspensions yielded 425 lesions; each of the 12 isolates induced between 12 and 26 lesions. The distribution of $SIZE_{max}$, PYC_{dens} , and $LatPYC_5$ for the 119 lesions obtained after ascospore inoculation is shown on Figure 2; pathogenicity components of the 12 isolates selected among 119 assessed after ascospore inoculation fell quite evenly within that distribution.

For each of the 12 selected isolates, latent period $LatPYC_5$ (Figure 3, Table 2) was statistically different after ascospore and pycnidiospore inoculation ($P = 0.041$). There was no difference either for lesion size $SIZE_{max}$ ($P = 0.123$) or pycnidium density PYC_{dens} ($P = 0.210$). $LatPYC_5$ was significantly longer after ascospore (557.4 ddpi) than pycnidiospore inoculation (501.9 ddpi). Means of $SIZE_{max}$ (13.4 mm² after ascospore inoculation vs. 11.4 mm² after pycnidiospore inoculation) and of PYC_{dens} (2.55 pycnidia.mm⁻² vs. 2.22 pycnidia.mm⁻²) were not significantly different.

The mean latent period $LatLES_{37}$ calculated from the cumulative number of sporulating lesions appeared after inoculation (when $PYC(t) \geq 0.05 \times PYC_{max}$) was 536.2 ddpi for ascospore inoculation and 472.7 ddpi for pycnidiospore inoculation (Figure 4). $LatLES_{37}$, which corresponds to the maximal rate of lesion appearance (time at which the mean number of new sporulating lesions appeared during one degree-day, $\Delta LES(t) / \Delta t$, is maximum), was reached, in average, 63.5 ddpi (3-4 days) later for ascospore than for pycnidiospore inoculation. The significance of the difference could not be tested statistically because of the heterogeneity of the two data sets used to calculate $LatLES_{37}$: on the one hand, 119 lesions induced by 119 different isolates after ascospore inoculations, on the other hand 425 lesions induced by 12 different isolates after pycnidiospore inoculations. This difference is, however, consistent with those obtained for $LatPYC_5$.

Latent period $LatLES_{37}$ after pycnidiospore inoculation was calculated similarly for each of the 12 selected isolates (Figure 5). The two measures of latent period were

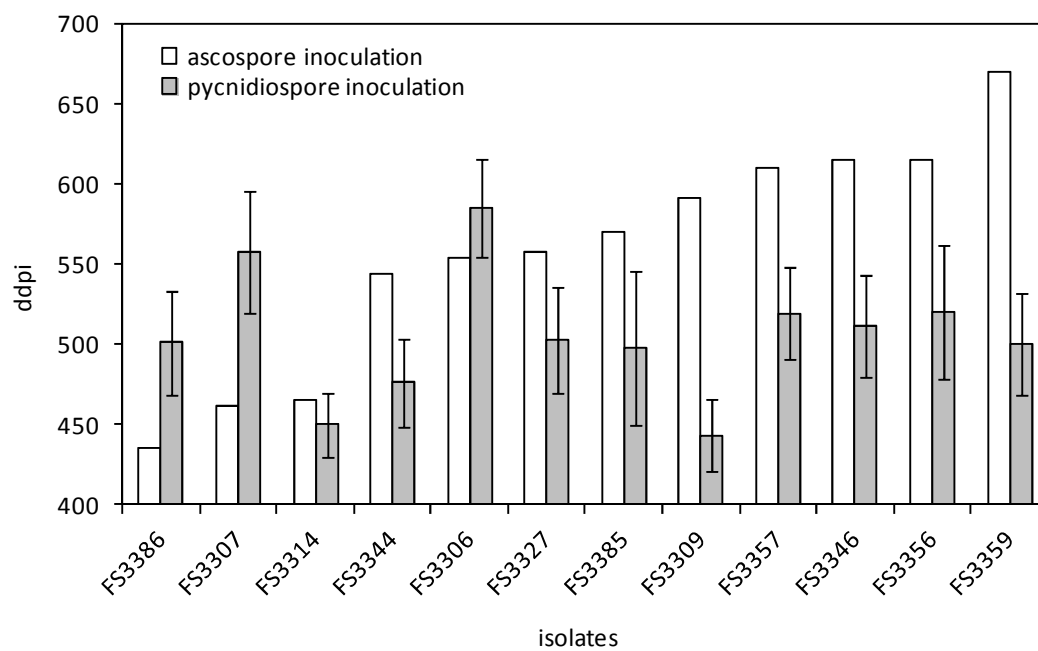


Figure 3 - Latent period (LatPYC₅) of 12 *Zymoseptoria tritici* isolates assessed after ascospore (white columns) and pycnidiospore (grey columns) inoculations on adult wheat leaves. Isolates are ranked in ascending order of LatPYC₅ assessed for ascospore inoculation; vertical bars represent standard deviation.

Table 2 - Effect of spore type (ascospores vs. pycnidiospores) on lesion size ($SIZE_{max}$), pycnidium density (PYCdens), and latent period (LatPYC₅) of 12 *Zymoseptoria tritici* isolates (see Table 1) inoculated on wheat cv. Soissons.

Variable	Factor	df	Sum of square	Mean square	F	P
$SIZE_{max}$	spore type	1	73.5	73.5	2.58	0.123
	residual	22	627.8	28.5		
PYCdens	spore type	1	0.90	0.90	1.67	0.210
	residual	22	11.90	0.54		
LatPYC ₅	spore type	1	15975.9	15975.9	4.70	0.041
	residual	22	74816.1	3400.7		

Table 3 - Effect of the origin of the inoculum (Grignon or Versailles) and of the presence or not of petroleum jelly on the trapping support on the latent period (LatPYC₅) assessed for 119 *Zymoseptoria tritici* isolates inoculated as ascospores (see Table 1) on wheat cv. Soissons.

Factor	df	Sum of square	Mean square	F	P
origine	1	3879.8	3879.8	0.503	0.480
petroleum jelly	1	271.9	271.9	0.035	0.851
origine × petroleum jelly	1	9323.4	9323.4	1.209	0.274
residual	115	886976.2	7712.8		

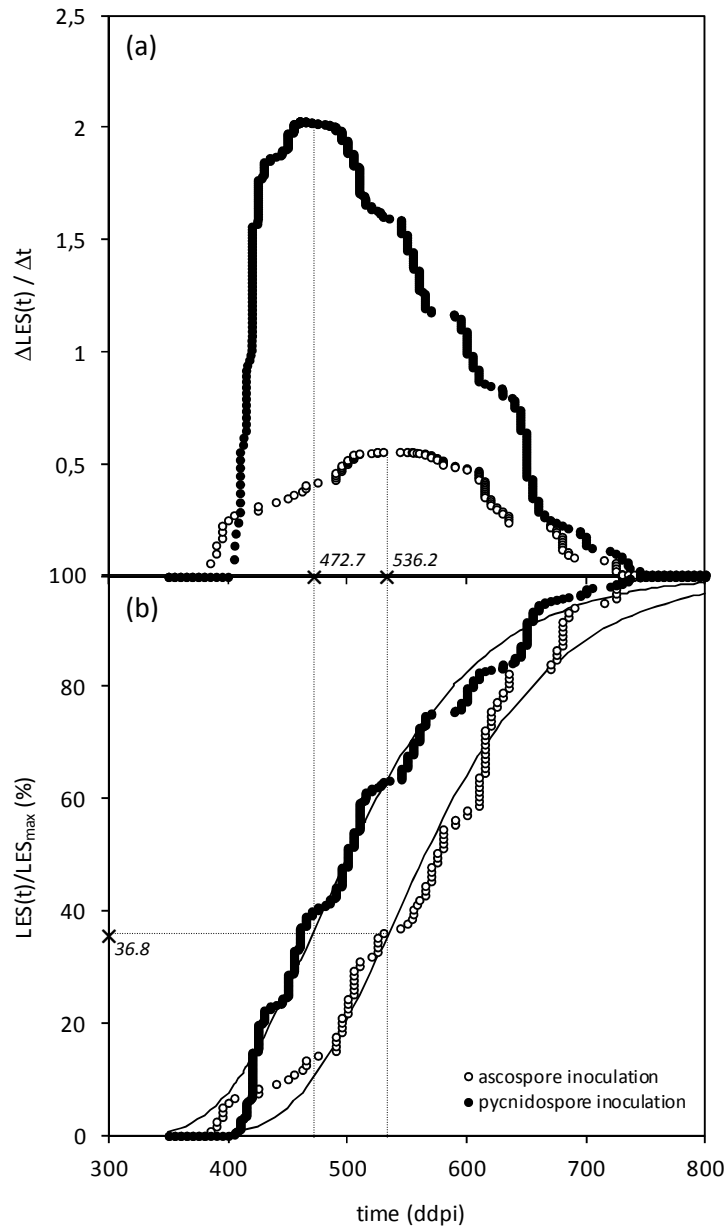


Figure 4 - (a) Mean number of new sporulating lesions appeared during a one degree-day period ($\Delta LES(t)/\Delta t$) after inoculation of 100 wheat leaves with 10 *Zymoseptoria tritici* ascospore suspensions (○) and after inoculation of 4×12 wheat leaves with pycnidiospore suspensions of 12 selected isolates (●). (b) Cumulative normalized number of lesions appeared after inoculation of 100 wheat leaves with ascospore suspensions (○) and after inoculation of 4×12 wheat leaves with pycnidiospore suspensions of 12 selected isolates (●).

Each point corresponds to a sporulating lesion. Lines correspond to the fitted curves (Gompertz model). Time is expressed in degree-days post-inoculation (ddpi). The mean latent period $LatLES_{37}$ (inflection point of the fitted curves) was 536.2 ddpi for ascospore inoculation and 472.7 ddpi for pycnidiospore inoculation.

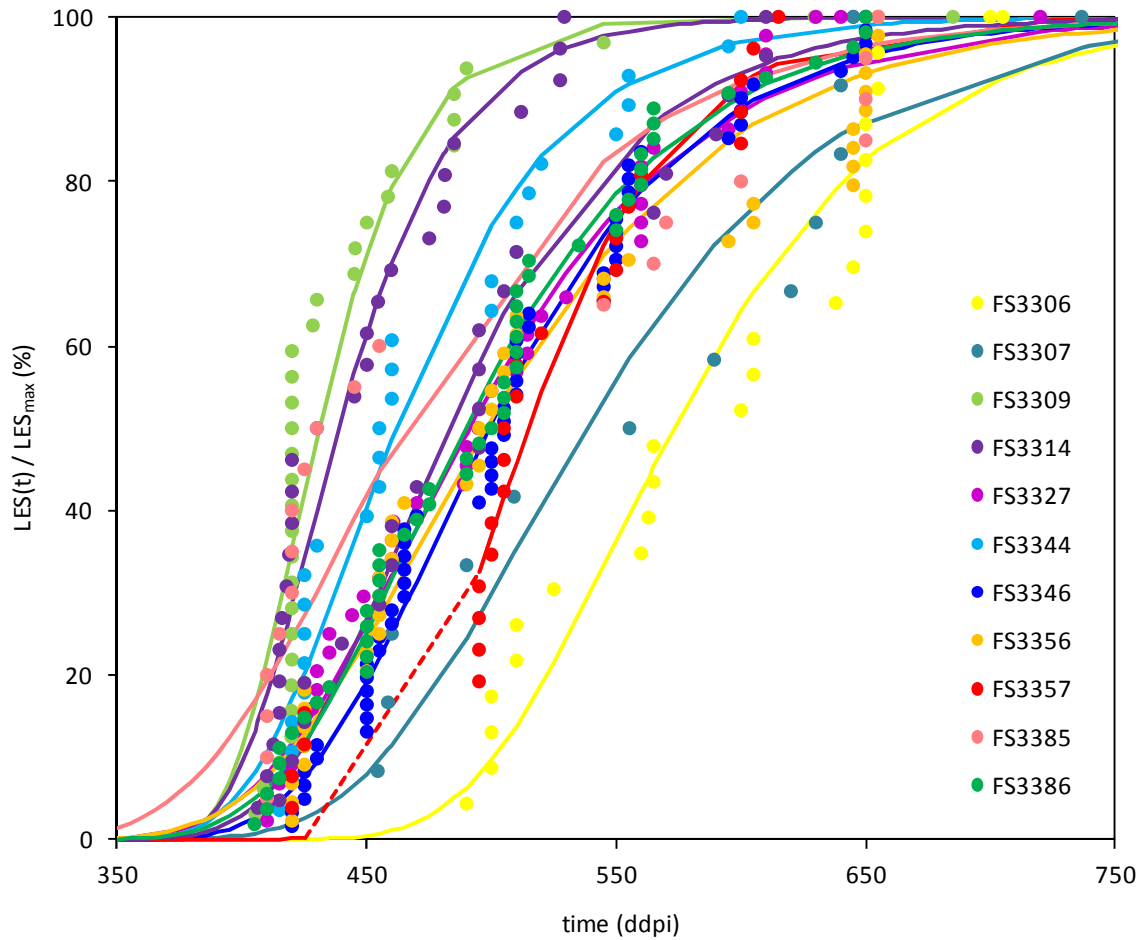


Figure 5 - Cumulative normalized number of lesions appeared after inoculation of 4×12 wheat leaves with pycnidiospore suspensions of 12 *Zymoseptoria tritici* isolates. Each point corresponds to the appearance of a new sporulating lesion. Lines correspond to the fitted curves (Gompertz model). Time is expressed in degree-days post-inoculation (ddpi).

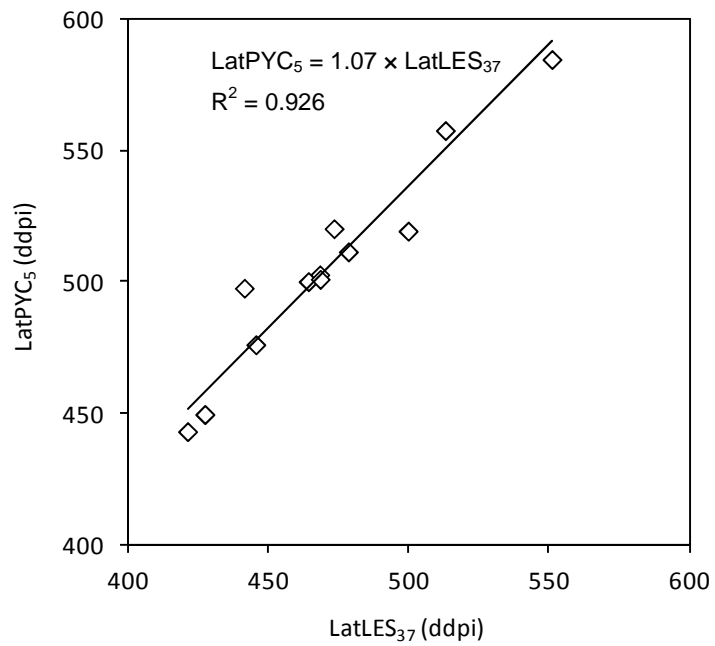


Figure 6 - Assessment of the latent period (ddpi, degree-days post-inoculation) of *Septoria tritici* blotch after inoculation of four wheat upper leaves with pycnidiospore suspensions (12 isolates, diamonds). LatLES₃₇ is the time elapsed from inoculation to the maximum rate of lesion appearance at the leaf scale; LatPYC₅ is the mean time elapsed from inoculation to the appearance of first pycnidia on the lesion.

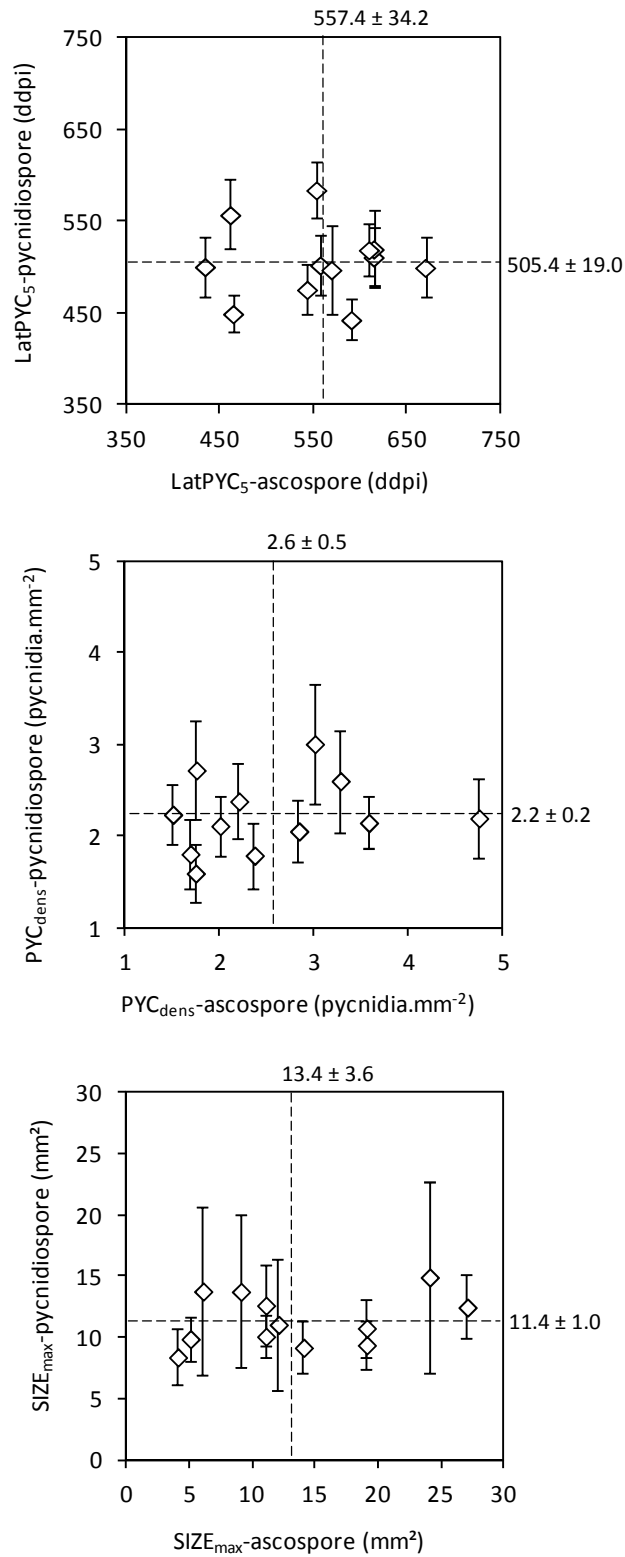


Figure 7 - Lesion size (SIZE_{max}), pycnidium density (PYC_{dens}), and latent period (LatPYC₅), assessed for 12 *Zymoseptoria tritici* isolates after ascospore and pycnidiospore inoculation. Each point corresponds to the mean of several values for pycnidiospore inoculation (vertical bars represent standard deviation) but to a single value for ascospore inoculation.

significantly correlated (Figure 6). The slope parameter close to 1 means that the average time of appearance of the first pycnidia is close to the average time at which the rate of sporulating lesion appearance is maximal.

LatPYC₅ calculated for the 119 lesions obtained after ascospore inoculation was not significantly affected either by the use of petroleum jelly (567.2 ddpi without petroleum jelly [68 lesions] vs. 570.8 ddpi with petroleum jelly [51 lesions]), or the origin of the isolates (567.2 ddpi for isolates originating from Versailles [19 lesions] vs. 566.8 ddpi for isolates originating from Grignon [100 lesions]) (Table 3). The difference in latent period between ascospore and pycnidiospore inoculations was neither due to an inhibitor effect of the petroleum jelly nor to differences in aggressiveness between pathogen populations of different origin.

The correlation between the mean values of lesion size (SIZE_{max}), pycnidium density (PYCdens), and latent period (LatPYC₅), assessed after ascospore and pycnidiospore inoculation for each of the 12 selected isolates is presented in Figure 7. For each pathogenicity component, the inter-isolates variability was higher after ascospore than pycnidiospore inoculation. The value obtained after pycnidiospore inoculation corresponds to the mean recorded on several lesions, while it corresponds to a single value recorded on one lesion after ascospore inoculation. Despite this experimental artifact, no relationship between pathogenicity components obtained after ascospore and pycnidiospore inoculation was found.

Discussion

In our experimental conditions, latent period of *Z. tritici* on adult wheat leaves was significantly longer after contamination with ascospores than with pycnidiospores, while no difference was established for lesion size and density of pycnidia. Differences in pathogenicity between asexual and sexual spores were hitherto reported for only a few other ascomycete fungi (Wood & Barbetti, 1977; Gilles *et al.*, 2001; Karolewski *et al.*, 2002; Mason & Huber, 2002; Li *et al.*, 2004; Trapero-Casas & Kaiser, 2007). As opposed to those studies, the high inter-individual variation in *Z. tritici* pathogenicity (Suffert *et al.*, 2013) prompted the use of asexual and sexual spores of the same genotypic origin, produced through a two-staged inoculation procedure. To the best of our knowledge, this is the first report of successful infection of wheat leaves with *Z. tritici* ascospores in controlled conditions and of the subsequent development of lesions. Moreover, it is also one of the first reports of infection

with *Z. tritici* asexual spores actually liberated by pycnidia born by a leaf lesion (pycnidiospores); in all reported infection experiments, the asexual spores were not pycnidiospores produced on infected leaves but conidia, obtained by clonal multiplication from yeast-like mycelium grown on artificial medium.

The difference in mean latent period between the two spore types, assessed as the maximum likelihood time elapsed between inoculation and the appearance of the first pycnidia ($\Delta\text{LatPYC}_5 = 55.5$ ddpi) was close to the difference assessed as the maximum likelihood time elapsed between inoculation and the appearance of the majority of the sporulating lesions ($\Delta\text{LatLES}_{37} = 63.5$ ddpi). This consistency is supported by the significant correlation between LatLES_{37} and LatPYC_5 assessed for the 12 selected isolates.

The longer latent period recorded after inoculation with sexual spores suggests that fungal growth that precedes the appearance of the new lesions is slower after inoculation with an ascospore than with a pycnidiospore. The early processes of infection (spore adhesion, germination, and penetration) might also be less efficient in ascospores than in pycnidiospores. Possible differences in infection efficiency - the probability that a spore deposited on a receptive host surface produces a lesion (Pariaud *et al.*, 2009) - could not be tested, however. Technical limitations prevented the precise assessment of inoculum concentration in the ascospore suspensions that is required for the accurate assessment of infection efficiency.

In contrast with our results, ascospores were reported to be more infectious than asexual spores in three ascomycete fungi. In *Leptosphaeria maculans* - the cause of blackleg disease on *Brassica* crops - ascospores caused more infections than pycnidiospores (Mason & Huber, 2002) and the latent period was shorter after infection with ascospores (Brunin & Lacoste, 1970; Alabouvette *et al.*, 1974). Moreover, germination and penetration, as well as the subsequent development of lesions, were much earlier in ascospores than in pycnidiospores (Li *et al.*, 2004). In *Pyrenopeziza brassicae* - the cause of light leaf spot of oilseed rape - a deposit of about 25 ascospores and about 700 pycnidiospores per leaf was required for infection (Karolewski *et al.*, 2002), confirming that ascospores of *P. brassicae* are more infective than conidia (Gilles *et al.*, 2001). Difference in latent period between the two spore types used for inoculation were not tested and it cannot be excluded that ascospores (mean latent period was 12.1 days) are less infective than conidia (mean latent period was 11.3 days; Karolewski *et al.*, 2002). In *Didymella rabiei* - the cause of Ascochyta blight of

Table 4 - Number of cells and volume of ascospores and pycnidiospores/conidia in four ascomycete fungi.

Fungus	Number of cells		Volume (μm^3) ^a	
	Ascospore	Pycnidiospore or conidia	Ascospore	Pycnidiospore or conidia
<i>Zymoseptoria tritici</i> ^b	2	4-8	50	210
<i>Pyrenopeziza brassicae</i> ^c	1-2	1	70	80
<i>Leptosphaeria maculans</i> ^d	6	1	1100	7
<i>Didymella rabiei</i> ^e	2	1-2	330	70

^a volume was approximated as $\pi/6 \times a \times b^2$ (ellipsoid) with a the mean length and b the mean width, whatever the shape of the spore.

^b a = 12.5 μm and b = 2.8 μm for ascospore (range, 10-15 \times 2.5-3.0 μm ; Shaner, 2010); a = 59.0 μm and b = 2.6 μm for pycnidiospore (range 20-98 \times 1.4-3.8 μm ; Shaner, 2010).

^c a = 14.5 μm and b = 3.0 μm for ascospore (range, 13.5-15.5 \times 2.5-3.0 μm ; Rawlinson *et al.*, 1978); a = 13.0 μm and b = 3.5 μm for pycnidiospore (range 10-16 \times 3.0-4.0 μm ; Rawlinson *et al.*, 1978).

^d a = 50 μm and b = 6.5 μm for ascospore (range, 30-70 \times 4.0-9.0 μm ; Smith & Sutton, 1964); a = 4.0 μm and b = 1.8 μm for pycnidiospore (range, 3.0-5.0 \times 1.5-2.0 μm ; Smith & Sutton, 1964).

^e a = 15.4 μm and b = 6.4 μm for ascospore (range, 13.5-17.3 \times 6.0-6.8 μm ; Nene, 1982); a = 13 μm and b = 3.3 μm for pycnidiospore (range, 10-16 \times 3.0-5.0 μm ; Punithalingam & Holliday, 1972).

chickpea - inoculation of plants with ascospores resulted in a significantly higher disease severity than with conidia, whose germination process was slower than in ascospores (Trapero-Casas & Kaiser, 2007). We propose to explain the inconsistency between the results obtained with *Z. tritici* and the three other fungi by differences in the size of the spores (Table 4). In *Z. tritici*, the spore volume is four times bigger for a pycnidiospore than for an ascospore. The volumes of the two types of spores are nearly equivalent in *P. brassicae*. In contrast, the spore volume is much bigger for an ascospore than for a pycnidiospore in *D. rabiei* ($\times 5$) and *L. maculans* ($\times 150$). The difference in latent period observed between the two spore types in those three fungi may be related to differential ability to cause infection: smaller spores, which also contain a smaller number of cells (Table 4), are likely to contain fewer nutrient reserves (Garrett, 1973); consequently, the time required for germ tube growth and penetration into host tissues may be longer. Ascospores of *D. rabiei* germinated more rapidly than conidia, which are smaller (Trapero-Casas & Kaiser, 2007). In the case of *Z. tritici*, we can assume that smaller ascospores germinated less rapidly than bigger pycnidiospores, and so needed more time to enter the host tissue. This assumption should be tested using microscopic observations.

Difference in latent period after ascospore and pycnidiospore infection could be related to different micro-climatic requirements. Temperature and wetness requirements for infection by two spore types have been rarely compared in ascomycete fungi (Karolewski *et al.*, 2002; Trapero-Casas & Kaiser, 2007). In our experiment, it is unlikely that wetness duration was a limiting factor because of the long wet period allocated after inoculation (72 h) with either of the spore types. The average temperature during the three weeks after inoculation was slightly higher for ascospores (18.9°C) than pycnidiospores (17.2°C). These temperatures are quite close to the thermal optimum of *Z. tritici* after conidial inoculation ($\sim 18^\circ\text{C}$; Bernard *et al.*, 2013); therefore, it is also unlikely that temperature was a limiting factor for any of the spore types.

Models of the development of *Septoria tritici* blotch that explicitly consider both ascospores and pycnidiospores, either all along the crop season (Eriksen *et al.*, 2001) or only at the onset of epidemic (Robert *et al.*, 2008), assume that the infection process after spore deposition is the same for both spore types. Our results suggest that the mean latent period during the very beginning of epidemics, when first lesions are mainly caused by wind-dispersed ascospores (Suffert *et al.*, 2011), is longer than during the spring epidemic stage, when secondary contaminations are caused by splash-dispersed pycnidiospores. This

difference can be increased by winter cold temperatures and the non-linearity of the thermal performance curve of *Z. tritici* (Shaw, 1990; Bernard *et al.*, 2013). According to our results, forecasting models used for timing fungicide sprays would be improved if the difference in latent period between the two spore types were considered. Spring infections are mainly caused by pycnidiospores splash-dispersed upwards in the wheat canopy; infections by airborne ascospores released by pseudothecia developing on wheat plants cannot be excluded (Duvivier *et al.*, 2013). Depending if the canopy is mainly contaminated by pycnidiospores or by ascospores, a 60-dd delay in the early dynamic of infection may alter the optimal schedule of fungicide spraying. For example, late preventive fungicides sprayed in late spring (from flag leaf visible Z37 to beginning of flowering Z60) may be more efficient in delaying or preventing infection by ascospores than by pycnidiospores.

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Chapitre IV

Analyses de populations locales de *Z. tritici* et inférence de l'origine de l'inoculum responsable des phases précoces d'une épidémie

Diversity and structure of *Zymoseptoria tritici* populations at a small spatiotemporal scale: overall genetic stability and sporadic signs of differentiation are not contradictory

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Abstract

By a molecular epidemiology approach, we investigated whether the diversity and the structure of *Zymoseptoria tritici* populations at a small spatiotemporal scale could help disentangling the influence of different local sources and types of inoculum on *Septoria tritici* blotch epidemics. Population genetic indexes derived from SSR markers analysis were used to assess changes in genetic structure and diversity between 26 populations (800 isolates) collected at different periods (from beginning to the end of epidemics) during four successive years (from 2009 to 2013), in two wheat fields contrasted for the origin of primary inoculum (with or without contaminated debris), and from pycnidiospores (asexual spores from leaf lesions) or ascospores (sexual spores from wheat debris). The genetic structure was stable over time (within and between years) and between plots. Diversity was very high in all populations. Two exceptions were detected. First, the differentiation between pairs of ascospore populations was lower than pycnidiospore populations, as suspected for a plant pathogen having dual sexual-asexual reproductive modes. Second, pycnidiospore populations collected during the whole spring 2012 were slightly, but significantly, more differentiated than populations of the previous years. Such signs of differentiation, related to the epidemiological context, allowed to reject the hypothesis that sexual secondary inoculum contributed significantly to the 2011-2012 epidemic, while it was not the case for the others. To conclude, genetic variation detectable by SSR genotyping, despite they were slight, were related to the amount and the type of inoculum: the overall genetic stability is not contradictory with sporadic signs of differentiation.

Keywords

Diversity, molecular epidemiology, population structure, *Mycosphaerella graminicola*, primary inoculum, *Septoria tritici* blotch, SSR, wheat debris.

Introduction

Understanding how fungal diseases start and develop and what the relevant biological and environmental determinants of epidemics are, is a challenging issue of crucial importance for disease management. Reducing the amount of inoculum that initiates an epidemic can be a successful means to limit the spread of an epidemic and its negative consequences on crop yield and quality (Van der Plank 1963; Strange 2003). Primary inoculum, defined as propagules or vegetative structures of a pathogen that cause initial rather than secondary outbreaks of disease (Shurtleff & Averre, 1997), is one key element of epidemics. Primary inoculum will influence the early stages of an epidemic and the disease intensity depends of its initial amount and conservation state: the more numerous are the living infectious propagules, the earlier (Jordan & Allen, 1984; Suffert & Sache, 2011) and more severe (Johnson 1990; McMullen et al., 2008) will be the epidemics. The nature of primary inoculum will also influence the genetic diversity of the pathogen populations that would thrive during the epidemic, causing a steadily adaptation to new cultivars or hosts (Ahmed et al., 1996; Pariaud et al., 2009). In addition, disease dispersal will be tailored by the nature of primary inoculum: highly dispersible propagules, with various dispersion modes may greatly enhance epidemics spread over a large territory (Purdy et al., 1985; Brown & Hovmøller, 2002; Fountaine et al., 2010). Finally, the period of production of primary inoculum has to be synchronized with the period of receptivity of host tissues. Since most fungal plant pathogens undergo vegetative reproduction as well as sexual reproduction, asexual and sexual inoculum can be jointly released during the onset of the epidemic and potentially involved in the initiation of the epidemic (Rutter et al., 1998; Suffert & Sache, 2011). Accordingly, disentangling the mechanisms involved in the early stages of epidemics and the effects of biotic and abiotic factors on such mechanisms is still a matter of debate among plant pathologists.

As asexual and sexual inoculum usually differ in their dispersal characteristics, e.g. wind vs. splash dispersal, the spatial patterns and genotypic diversity of pathogen populations are both useful for inferring the type of inoculum most significant in an epidemic period (Milgroom & Peever, 2003). The observation and the modeling of the spatio-temporal dynamics of the propagules suspected to act as primary inoculum can allow the identification of the sources of primary inoculum (Rieux et al., 2014). Such approaches, however, add little information on the genetic structure of the pathogen population and its evolution during the

build-up of primary inoculum. Population genetics helps understanding the evolutionary processes involved in the establishment and maintenance of genetic variation within and among populations, focusing on changes of multilocus genotypes or allele frequencies (McDonald & Linde, 2002; Abang et al., 2006). Therefore, population genetics could help revealing when the primary inoculum is produced and contributes to epidemics onset (Zwankhuizen et al., 1998; Shah et al., 2001; Milgroom & Peever, 2003). Primary inoculum can be constituted of new genotypes produced through sexual reproduction and/or of airborne propagules of remote origin, which may modify allele frequencies and the subsequent population structure. Alternatively, primary inoculum can also be constituted of local, related genotypes, which would not deeply modify the population structure.

Epidemiological studies aiming to infer dispersal capacities during intra- and inter-epidemics periods can benefit from molecular epidemiology approaches. Such approaches, that combines population genetics and dynamics, have established the role of the different sources of primary inoculum in the initiation of fungal plant diseases, such as *Stagonospora nodorum* (Shaw, 1999), *Rhynchosporium secalis* (Fontaine et al., 2010) and *Fusarium* sp. (Landschoot et al., 2011) on wheat, *Melampsora larici-populina* on poplar (Xhaard et al., 2012), *Stemphylium vesicarium* (Rossi et al. 2008) on pear, *Puccinia striiformis* f. sp. *tritici* on wheat (Ali et al., 2014) and *Ascochyta rabiei* on chickpeas (Peever et al., 2004). The influence of the source of primary inoculum on the subsequent genetic structure of the fungal populations, during the early stages of an epidemic, has yet to be assessed.

Septoria tritici blotch causes up to 30-40% yield loss (Eyal et al, 1987) on wheat (*Triticum aestivum*) in most wheat-growing areas worldwide. The disease is caused by the hemibiotrophic, heterothallic, ascomycete fungus *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*, anamorph *Septoria tritici*; Quaedvlieg et al., 2011). Among the means of disease control, crop rotation and stubble management are expected to decrease the amount of primary inoculum (Eyal, 1999). Ascospores (Sanderson, 1972; Halama, 1996), ejected from contaminated debris from the previous wheat crop and wind-dispersed over long distances, constitute the main source of *Z. tritici* primary inoculum while pycnidiospores, splash-dispersed over shorter distances, are the main source of secondary inoculum during the growth period of the wheat crop (Shaw & Royle, 1989; Suffert et al., 2011). However, ascospores and pycnidiospores can be jointly involved into different stages of the epidemic development. In a wheat monoculture system, when contaminated debris is not completely buried, pycnidiospores can theoretically contribute to initiate the epidemic (Suffert et al.,

2011). In spring, secondary infections can be also induced by ascospores (Duvivier et al., 2013) released either from old, still infectious debris located in distant plots or from infected wheat plants located in the same plot. Suffert & Sache (2011) showed in a field experimental study that an epidemic developed faster in a plot with debris, releasing ascospores, than in a plot without debris; the genetic structure of the *Z. tritici* population in the two epidemics has not yet been investigated. The level of genetic recombination in *Z. tritici* populations and the relative importance of pycnidiospores and ascospores in the epidemic development are difficult to obtain experimentally (Zhan et al., 1998; 2000); in this context, results of simulations of a mathematical model including the effect of the two spore types on the epidemic development suggested that sexual recombination will affect the genetic composition of the population during a growing season (Eriksen et al., 2001). Investigation on the genetic diversity and structure of populations of *Z. tritici* at a single date during annual epidemics were mainly carried out at a large geographical scale (Zhan et al., 2002; 2003; Razavi & Hughes, 2004; Abrinbana et al., 2010; El Chartouni et al., 2011; Boukef et al., 2012), but rarely at a local (field) or very local (plant or leaf) scale (Chen et al., 1994; Linde et al., 2002; El Chartouni et al., 2012). Despite the potential differences in the nature and the amount of inoculum within an epidemic, the sampling period of *Z. tritici* populations was rarely considered as a factor that affects their genetic structure. Populations exhibited a very high genetic diversity and no structure at the continental and regional scales (Schnieder et al., 2001; Linde et al., 2002), except in the studies of Chartouni et al. (2011; 2012) and Abrinbana et al. (2010). Finally, there is still too few information on the evolution of *Z. tritici* populations at a local scale, which is the spatio-temporal scale relevant to the understanding of the early stages of disease epidemics and the effective influence of primary inoculum on the population structure and diversity.

The objective of this study was to investigate whether the evolution of *Z. tritici* populations at a small spatiotemporal scale could help disentangling the influence of different sources and natures of primary inoculum on Septoria tritici blotch epidemics. To this aim, we developed a molecular epidemiology approach and addressed the following questions: (1) How do *Z. tritici* populations locally evolve during several successive epidemics and which factors may shape the structure of these populations? (2) Does the origin of the primary inoculum modify local population diversity and the occurrence of sexual reproduction? We assessed and analyzed the changes in the genetic structure and diversity of several *Z. tritici*

populations collected at a local scale during three successive yearly epidemics in two wheat fields, contrasted in terms of debris management that is for the origin of primary inoculum.

Materials & Methods

Experimental design and sampling

A four-year field experiment was carried out from 2009 to 2013 at the INRA Grignon experimental station (France, 48°51'N, 1°58'E), on the basis of the field design described by Suffert & Sache (2011). A 20 × 100 m monoculture plot (noted D+) was cropped with winter wheat (cv. Soissons), without fungicide application since 2007, during the 2009-2010, 2010-2011, 2011-2012 cropping seasons and beginning of the subsequent 2012-2013 campaign. Debris of the previous wheat crop was left on the soil surface. Another 40 × 100 m plot (D₀), not cultivated in wheat the previous year, was cropped with winter wheat each of the 2009-2010, 2010-2011, and 2011-2012 seasons. The D+ and D₀ plots were contiguous in season 2010-2011, while they were located 300 m apart during 2009-2010 and 2011-2012 seasons. In the D₀ plot, no debris was present over the three years of the study so that *Z. tritici* primary inoculum infecting this plot was assumed to have a distant origin. Samples originated from pycnidiospores were collected from wheat leaves infected with *Z. tritici* from D+ and D₀ plots simultaneously every one or two months from October 2009 to December 2012. Fungal isolates were obtained by placing the infected leaves in a plastic box with a wet paper, kept at 18°C overnight to promote cirrus exudation. One cirrus per pycnidia and per leaf lesion was taken and cultured in a single Petri dish filled with PDA medium (Potato Dextrose Agar 39 g.L⁻¹) for five days. This led to the collection of 685 pure isolates. Samples collected at close dates were grouped together according to the epidemic stage in order to constitute 23 groups of 16-84 individuals (hereafter called "populations" for the sake of simplicity; numbered P1 to P29). Consequently, four groups were determined for cultural seasons 2010-2011 and 2011-2012 (Figure 1): "beginning of epidemic period" (from October to January, which corresponds to autumn and to the first weeks of winter, where wheat was beginning tillering; populations P02, P05, P09, P13, P17, P23, and P29), "intermediate epidemic period 1" (from February to late-March, which corresponds to the end of winter and the beginning of spring; P10, P14, P18, and P24), "intermediate epidemic period 2" (from April to May, which corresponds to spring period; P03, P06, P11, P15, P19, and P25) and "end of epidemic period" (from late-May to mid-July, which corresponds to the end of spring and the first of

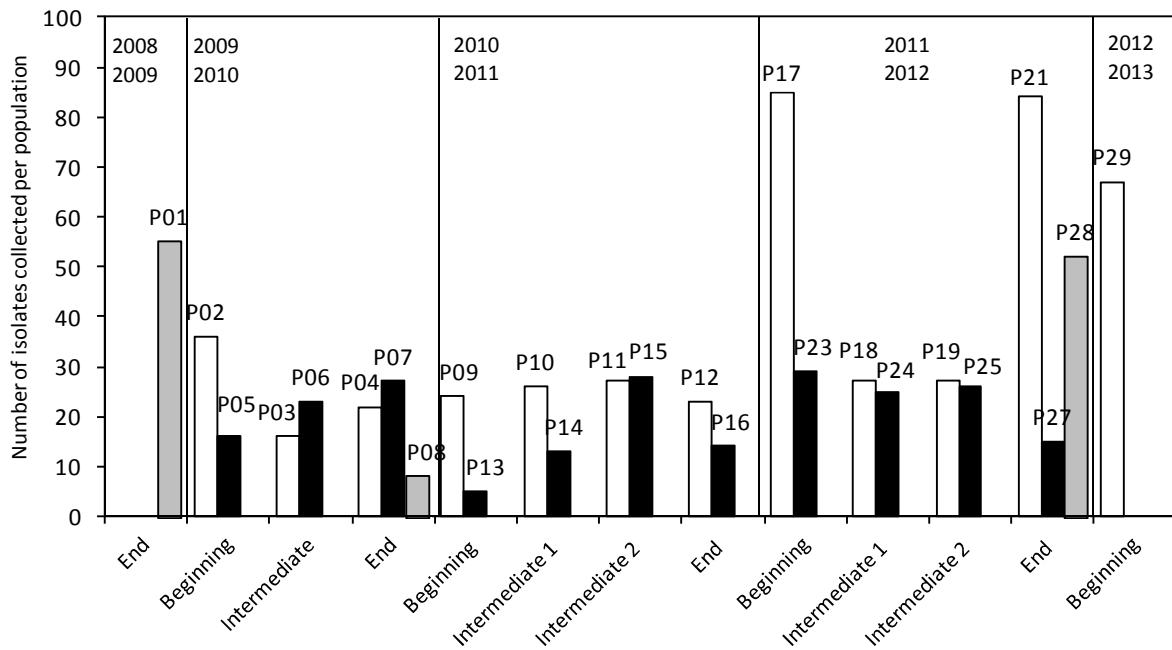


Figure 1 - Populations of *Z. tritici* (800 isolates) collected in two wheat plots (D+, with debris; D₀, without debris) during three cultural seasons (2009-2010, 2010-2011 and 2011-2012), in Thiverval-Grignon, France. The 800 isolates were grouped according to the epidemic period (beginning, i.e. from late-November to late-January; intermediate 1, i.e. February and March; intermediate 2, i.e. from April and May; end, i.e. from early-June to mid-July). White columns: pycnidiospore populations from plot with debris (D+); black columns: pycnidiospore populations from plot without debris (D₀); gray columns: ascospore populations from plot with debris (D+).

summer, when wheat reaches the adult mature stage; P04, P07, P12, P16, P21, and P27). For the 2009-2010 season, only three groups were constituted because of low disease severity at the "intermediate period", which corresponds to only one collection date in March 2010, the "beginning period" and "end period" being similar as previously described.

Populations originated from ascospores (hereafter called "ascospore populations") were collected after the inter-epidemic period from wheat debris bearing perithecia in the D+ plot. Wheat debris were kept on plastic boxes and wetted at 18°C overnight. The next day, debris were cut in small pieces of 10 g and spread on fresh moist boxes. Petri dishes filled with PDA medium were put 1 cm above debris in each box. Then, moist boxes were kept in the dark for at least 6h at 18°C and then were covered up and incubated in the same conditions until colony formation, which were then isolated. This led to the collection of 115 isolates, organized into three populations (P01, P08, and P28).

In total, 800 pure strains (Figure 1) were isolated and organized into 26 populations according to the type of spores from which the isolates come from (pycnidiospore or ascospore), the origin (plot with or without debris), and the epidemic period (beginning, intermediate 1, intermediate 2, end). Isolates were kept at -80°C in a 1:1 glycerol/water solution until further use.

Molecular procedures

For each isolate, DNA was extracted from 50 mg of fresh fungal material, scrapped from five days of culture on PDA medium in the dark at 18°C. DNA was extracted in an automated system (Biorobot 3000, Qiagen), with the DNeasy adapted kit (Qiagen). All 800 isolates were genotyped for eleven microsatellite markers (St2, St3A, St3C, St4, St5, St6, St7, St9, St10, St11 and St13 ; Gautier et al., 2014) organized in two multiplex PCRs. Automatic allele recognition and annotation was carried out to name homogeneously alleles for each locus (Beckmann Coulter CEQ 8000 software; Gautier et al., 2014). Five hundred and eighty five isolates were completely genotyped for the 11 SSR (Single Sequence Repeat) markers, while the remaining isolates (27% of the total dataset) had only one or two missing allele values, homogeneously distributed along all SSRs. The mating-type of 739 isolates was determined by PCR (Waalwijk et al., 2002); eight per cent of the isolates were not genotyped for mating type, because of technical failure or contaminations during PCR assays.

Analyses of population subdivision without *a priori* assumptions

Population subdivision without *a priori* assumptions was investigated by the Bayesian clustering method implemented in the STRUCTURE program (Pritchard et al., 2000). This model-based algorithm assumes linkage equilibrium within inferred genetic clusters, therefore is in principle appropriate for species experiencing regular recombination. We ran STRUCTURE with the admixture model and correlated allele frequencies. Burn-in length was set at 500,000 Markov Chain Monte Carlo iterations. The burn-in period was followed by a run phase of 1,000,000 iterations (El Chartouni et al., 2011), with the number of clusters K ranging from 1 to 10 with 10 independent replicates for each value of K . The amount of additional information explained by increasing K was determined by calculating the ΔK statistic (Evanno et al., 2005) with STRUCTURE HARVESTER (Earl & von Holdt, 2012, Belkhir et al., 1996-2004).

Three STRUCTURE analyses were carried out independently, to test the three factors that can be suspected to structure the dataset, namely i) the collection time, through the three cultural seasons divided into three or four epidemic periods, ii) the origin of the isolates (plot with or without debris), and iii) the type of spores (from which the isolates come from, i.e. ascospore or pycnidiospore). A first set of analyses was carried out in order to test, for each cultural season separately (2009-2010, 2010-2011, 2011-2012), the effect of epidemic period on the structure of pycnidiospore populations collected in the plot with debris (P02-P03-P04, P09-P10-P11-P12, and P17-P18-P19-P21, respectively); an analysis grouping together the three year datasets, adding P29 (2012-2013), was also carried out overall in order to test the year effect. A second set of analysis was carried out to test, for each cultural season separately (and then overall), the structuring effect of the origin of the isolates (plot with or without debris) on the structure of pycnidiospore populations (P02-P03-P04-P05-P06-P07, P09-P10-P11-P12-P13-P14-P15-P16, and P17-P18-P19-P21-P23-P24-P25-P27, respectively). The last analysis was carried out to test the structuring effect of the type of spores on the complete dataset (adding P01-P08-P28).

Analyses of population subdivision with *a priori* assumptions

A 3D Principal Component Analysis (PCA) was achieved using GENETIX (Belkhir et al., 1996-2004) on multilocus genotypes categorized by populations (as defined in Figure 1) to investigate their relationships. At last, hierarchical analyses of molecular variance (AMOVA)

implemented in ARLEQUIN V3.5 (Excoffier & Lischer, 2010) were performed in order to investigate the relative contributions of time (year and epidemic period), of origin of the isolates, and of the type of spores to the partitioning of genetic variance. Each factor was tested individually using *a priori* populations (as defined in Figure 1) and performing one or two factor AMOVAs.

Genetic variability, differentiation between populations and occurrence of sexual reproduction

Pairwise Weir & Cockerham's F_{ST} index (Weir & Cockerham, 1984) were calculated between pairs of populations defined in Figure 1, using GENEPOP v4.1 (Raymond & Rousset, 1995). The unbiased fixation index F_{ST} constitutes a common measure of the degree of genetic differentiation, and was first defined by Falush et al. (2003), which by definition lies between zero (total panmixia) and one (population have gone to fixation for different alleles). F_{ST} can be thought as the proportion of genetic diversity due to allele frequency differences among populations or as the correlation between alleles within populations relative to the entire population. Significance of F_{ST} values was calculated after 1000 randomizations.

GENETIX (Belkhir et al., 1996-2004) was used to estimate over time within-population genetic variability on the basis of genic diversity calculated as multilocus observed heterozygosity (H_E), and allele richness (A_r , mean number of alleles per locus), in all the populations. *t*-tests for paired samples were used to check significant differences between D+ and D₀ plots over time (5% confidence level).

To assess the occurrence of sexual reproduction of *Z. tritici* populations over three cultural seasons, the number of unique multilocus genotypes (G) and the clonal fraction (1-G/N) were calculated using MULTILOCUS v1.3B (Agapow & Burt, 2001). This software was also used to estimate multilocus linkage disequilibrium overtime, as the r_D index. This is corrected for the number of loci considered, and goes from 0 (all individuals are genetically different and multilocus linkage are broken) to 1 (all individuals are clones). r_D significance was established by comparing the observed values with the distributions obtained by 1000 randomizations (Agapow & Burt, 2001). At last, to assess the possibility of recombination by sexual reproduction, we calculated the ratio between mating-type alleles (Mat 1-1/Mat 1-2). χ^2 tests were performed to determine whether the frequencies of the two mating types within different populations departed from the null hypothesis of a 1:1 ratio. *t*-tests for paired

samples were also performed to check significant differences over time between D+ and D₀ plots (5% confidence level) for all indexes.

Results

Population partitioning

Bar plots of the three independent STRUCTURE analyses carried out to evaluate population subdivision with the admixture model and without using prior assumptions about time (year and epidemic period), origin of isolates (plot with or without debris), and type of spores (ascospore or pycnidiospore), were given in Figure 2. The three cultural seasons (2009-2010, 2010-2011, 2011-2012) and the adjacent epidemic periods (end of cultural season 2008-2009, P01; beginning of cultural season 2012-2013, P29) were presented together. In the first set of analyses, pycnidiospore populations from the D+ plot were analyzed for each cultural season (Supplementary Table 1) and then overall (Supplementary Table 1; Figure 2A). The rate of change in the log probability of data between successive K values (ΔK) exhibited a mode at $K = 3$ for the 2009-2010 season, at $K = 2$ for the 2010-2011, 2011-2012 cultural seasons, and at $K=7$ for the overall dataset. Whatever the epidemic period considered in each cultural season (year), individuals were hardly assigned to one of these K clusters (no individuals assigned to one cluster with a posterior probability > 0.5), their posterior probability being equally shared between the seven clusters ($0.1426 < \text{overall mean } P < 0.1431$), suggesting that neither the epidemic period nor the cultural season deeply structured the pycnidiospore populations. In the second set of analyses, pycnidiospore populations from the D+ and D₀ plots were analyzed for each cultural season (Supplementary Table 1) and then globally (Supplementary Table 1; Figure 2B). Modes at $K = 3$ for the 2009-2010 season and at $K = 2$ for the 2010-2011, 2011-2012 seasons and for the overall dataset were exhibited. Again, the posterior probability of individuals was equally shared between the two clusters ($0.498 < \text{overall mean } P < 0.502$). Only 0.15 % of the individuals were assigned to one cluster with a posterior probability greater than 0.6, whatever the epidemic period, suggesting again that the origin of isolates (D+ or D₀ plots) did not highly structure the dataset. At last, after addition of ascospore populations to the previous dataset in the third STRUCTURE analysis (Supplementary Table 1; Figure 2C), a mode at $K = 3$ and posterior probabilities still equally shared ($0.332 < \text{overall mean } P < 0.334$) were exhibited; thus, there was no structuring effect of the type of spores.

As no population structure was detected without *a priori* approach, the dataset was explored using *a priori* populations defined in Figure 1. The relationships between the 26 populations is presented in a three dimensional PCA graph (Figure 3). All populations had a close inertia centre position regarding the three axes and constituted one single tight cloud, except four populations which were distinguished from the central cloud: P09 (beginning of season 2010-2011 in D+ plot), P18 (first intermediate period of season 2011-2012 in the D+ plot), P25 (second intermediate period of season 2011-2012 in the D₀ plot), and P27 (end period of season 2011-2012 in the D₀ plot).

We examined the differentiation between and among populations collected from pycnidiospores and from ascospores by calculating pairwise F_{ST} (Figure 4). The overall mean F_{ST} was 0.016, with 22.5% of individual F_{ST} values being significant. This mean was chosen to calculate the proportions of significant F_{ST} values greater than this threshold, between and among pycnidiospores and ascospores populations (Table 1). Ascospore populations arise exclusively from recombination, while pycnidiospore populations are supposed to derive from ascospores populations after a variable number of clonal reproduction cycles. Differentiation between pairs of pycnidiospore populations was low, with a mean F_{ST} value equal to 0.017 but 24.9% of significant pairs greater than the overall mean F_{ST} . By contrast, the differentiation was even lower between pairs of ascospore populations (mean $F_{ST} = -0.003$) and between pairs of ascospore and pycnidiospore populations (mean $F_{ST} = 0.010$). This lower differentiation is confirmed by the null or lower proportion of significant F_{ST} values greater than our threshold (Table 1). Scoring the number of significant pairwise comparison including each given populations enabled to highlight the pycnidiospore population collected in the D₀ plot at the end of season 2010-2011 (P16), and most of the pycnidiospore populations collected at the end of season 2011-2012 (P18, P19, P25, P21 and P27) (Supplementary Table 2 and Figure 4). These populations were involved in 13-14 significant pairwise comparisons, whereas all the others exhibited only 0-7. P18, P25 and P27 have been already distinguished with the 3D PCA. These results constitute a body of inter-related evidence that populations collected during the whole spring 2012 are slightly more differentiated than populations from seasons 2009-2010 and 2010-2011.

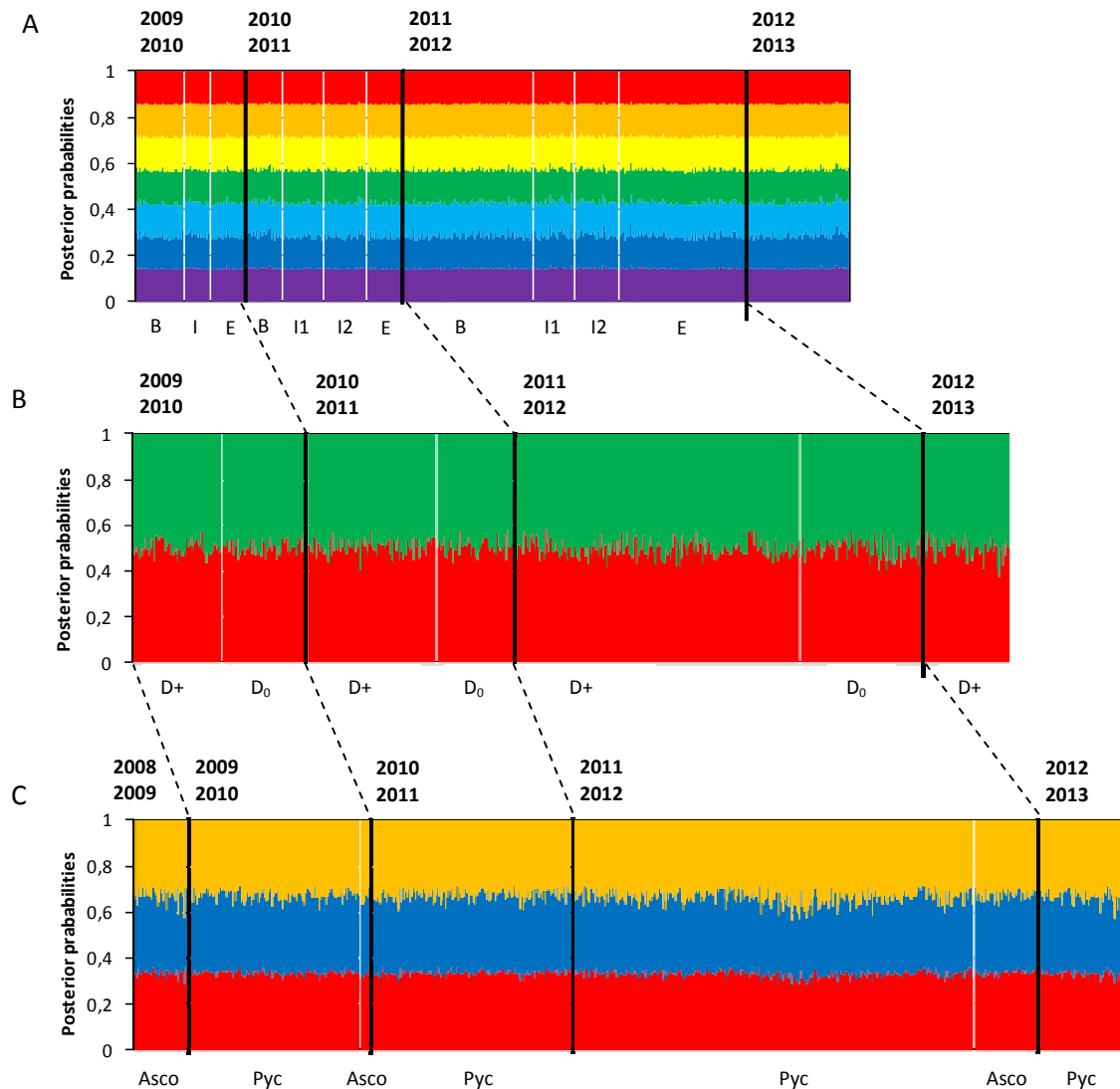


Figure 2 - Genetic subdivision of the 800 *Z. tritici* isolates collected during three cultural seasons (2009-2010, 2010-2011 and 2011-2012), in Thiverval-Grignon, France. Colors represent membership of the individuals into K clusters, as defined after STRUCTURE analysis, aiming to test three structuring hypothesis. A - Test of the epidemic period ($K = 7$; 460 isolates; letters indicate the different epidemic periods, B: beginning; I1 and I2: intermediate; E: end). B - Test of the origin of isolates ($K = 2$; 685 isolates; D+: plot with debris, D₀: plot without debris). C - Type of spores ($K = 3$; 800 isolates; Asco: ascospores; Pyc: pycnidiospores). For each barplot, the vertical black lines delimit the different cultural seasons and the vertical white lines delimit the modality of the second studied factor (the epidemic period in A; the origin of isolates in B; the type of spores in C).

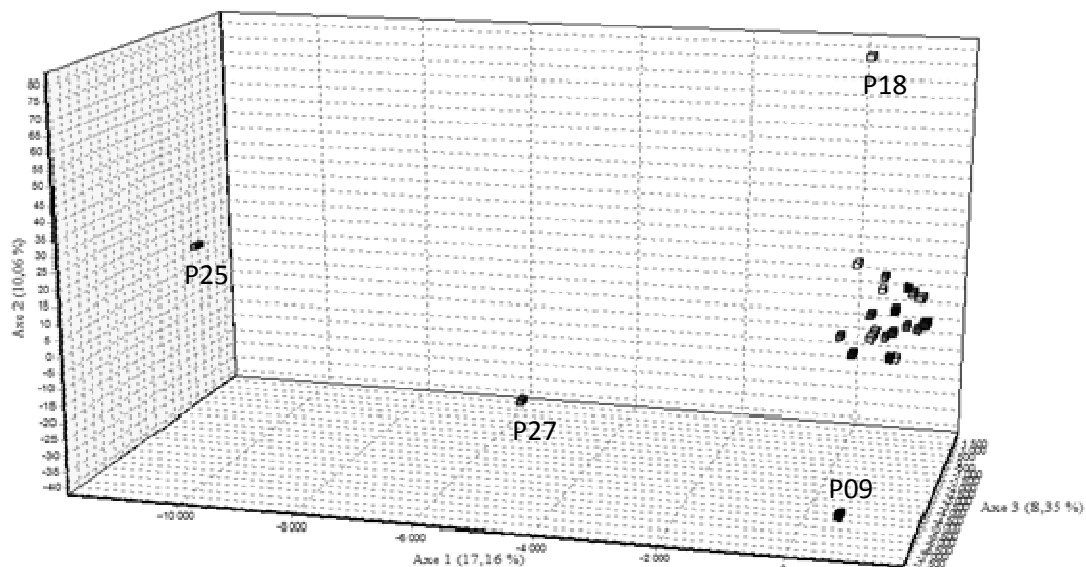


Figure 3 - Principal components analysis (PCA) of the genotypic proximity of 26 *Z. tritici* populations (800 isolates; see Figure 1) collected during three cultural seasons (2009-2010, 2010-2011 and 2011-2012). Each of the 26 populations is represented by a square. Outlier populations: P09 (beginning of season 2010-11 in D+ plot), P18 (first intermediate period of season 2011-2012 in the D+ plot), P25 (second intermediate period of season 2011-2012 in the D₀ plot), and P27 (end period of season 2011-2012 in the D₀ plot).

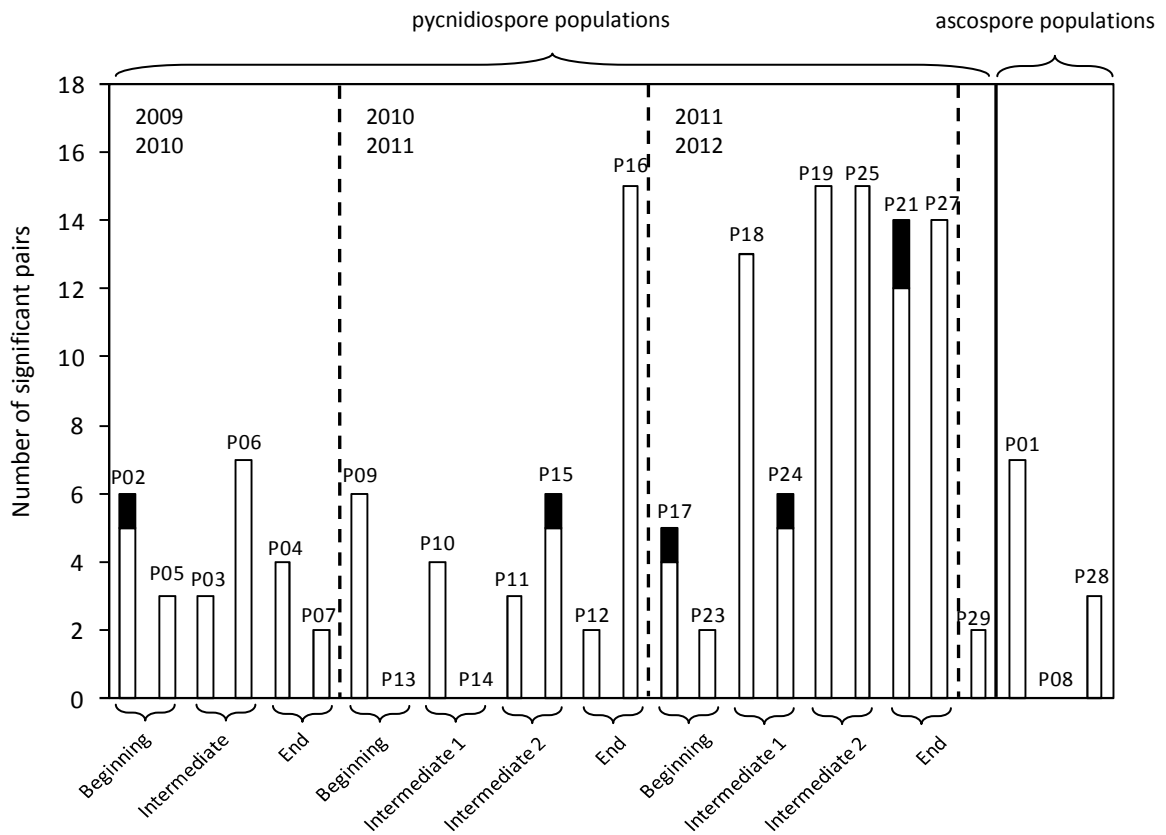


Figure 4 - Comparison of number of pairwise F_{ST} exhibiting significant values ($P < 0.05$) for the 26 *Z. tritici* populations according to the type of spores (pycnidiospores, ascospores), to the cultural season (2009-2010, 2010-2011, 2011-2012), and to the epidemic period (beginning, intermediate, end). Black bars indicate the number of pairs with significant F_{ST} values lower than the mean overall F_{ST} (0.016), whereas white bars are greater than this threshold (see Supplementary Table 2).

Table 1 - Differentiation between populations estimated by pairwise F_{ST} values (see Supplementary Table 2). The percentages of pairwise comparisons where F_{ST} were significant ($P < 0.05$) and greater than the overall mean F_{ST} (0.016) were calculated for each comparison of type of spore populations.

	Number of pairs	Mean F_{ST}	% of significant $F_{ST} >$ overall mean F_{ST}
Overall	325	0.016	22.5%
Pairwise F_{ST} between pycnidiospore populations	253	0.017	24.9%
Pairwise F_{ST} between ascospore and pycnidiospore populations	69	0.010	14.5%
Pairwise F_{ST} between ascospore populations	3	-0.003	0%

We then examined the partitioning of genetic variation among the different potential factors shaping *Z. tritici* population subdivision (year and epidemic period, origin of isolates, type of spores) by hierarchical analyses of molecular variance (AMOVA) (Tables 2 and 3; Supplementary Table 3).

A first two factor AMOVA, performed to test the effect of year (cultural season) and epidemic period nested within cultural season in each plot, separately, indicated that variation within epidemic periods accounted for most of the molecular variance (98-99%; $P < 0.002$; $0.012 < F_{ST} < 0.020$). Variation among epidemic periods within cultural seasons accounted only for 1.7-2.1% of molecular variance but was still significant ($P \leq 0.005$). This result shows that genetic variation among populations was, overall, very slightly affected by the epidemic period. Secondly, a set of one factor AMOVAs performed to test the effect of the origin of isolates nested within the epidemic period (Table 2), showed that, for all epidemic periods, the variation within plots accounted for most of the molecular variance (97.6-99.6%). Nevertheless, variation among plots was significant for the intermediate epidemic periods 1 and 2, even if it explained very small parts of molecular variance (1.9-2.4%; $P < 0.034$; $0.019 < F_{ST} < 0.024$). The genetic variation of the populations was therefore slightly, although significantly, affected by the origin of isolates (plot D+ or D₀) only between January and April, when the amount of airborne ascospores is usually decreasing, compared to the beginning and the end of the epidemics. A third set of AMOVA analyses (Table S3) was performed to test the effect of the type of spores on the genetic structure of populations at relevant epidemic periods (end/beginning) and test the hypothesis of temporal continuity. We compared the genetic variation between pycnidiospore populations collected at the end of each epidemic (P04, P21) and ascospore populations present on debris of the corresponding crop collected at the beginning of the subsequent epidemic (P08, P28, respectively) in D+ plot, and then in D₀ plot (P07, P27, respectively; Table S3A). The variation within plots accounted for most molecular variance (96.7-100%). Variation among plots was not significant, except for the P27-P28 comparison (3.4%; $P < 0.05$; $F_{ST} = 0.033$); the difference in the genetic structure between P27 and P28 was low but significant. Then, we tested the hypothesis that early infections in plot D+ were mainly caused by local inoculum, that is, built-up by ascospores ejected from debris of the previous wheat crop. Each ascospore population collected from wheat debris at the beginning of epidemics (P01, P08, P28) was compared with the pycnidiospore population collected during the same period in D+ plots (P02, P09, P29, respectively), and with the pycnidiospore population collected in D₀ plots

Table 2 - Hierarchical analyses of molecular variance (AMOVA) with cultural season and epidemic period nested within cultural season as grouping factors, for D+ (upper panel) and D₀ (lower panel) plots.

	df	Sum of squares	Variance components	% of variation	<i>P</i> -value	Fixation index
Plots with debris (D+)						
Among cultural seasons	2	3.6	-0.01	-0.56	0.935	-0.005
Among epidemic periods	8	23.6	0.032	1.71	0.001	0.017
Within cultural seasons						
Within epidemic periods	386	714.6	1.851	98.85	< 0.001	0.012
<i>Total</i>	<i>396</i>	<i>741.7</i>	<i>1.873</i>			
Plots without debris (D₀)						
Among cultural seasons	2	4.7	-0.0003	-0.02	0.349	< 0.001
Among epidemic periods	8	18.2	0.034	2.05	0.005	0.020
Within cultural seasons						
Within epidemic periods	210	341.6	1.626	97.97	0.002	0.020
<i>Total</i>	<i>220</i>	<i>364.5</i>	<i>1.660</i>			

Table 3 - Hierarchical analyses of molecular variance (AMOVA) with collected plots (D+ or D₀), contrasted for the origin of isolates, as grouping factors, for each epidemic period.

	df	Sum of squares	Variance components	% of variation	P-value	Fixation index
Beginning of the epidemics						
Among plots	1	2.4	0.007	0.37	0.183	0.003
Within plots	193	370.4	1.919	99.63		
<i>Total</i>	<i>194</i>	<i>372.8</i>	<i>1.926</i>			
Intermediate period 1						
Among plots	1	2.8	0.029	1.92	0.034	0.019
Within plots	89	133.3	1.498	98.08		
<i>Total</i>	<i>90</i>	<i>136.1</i>	<i>1.777</i>			
Intermediate period 2						
Among plots	1	4.3	0.038	2.37	0.002	0.024
Within plots	145	225.7	1.556	97.63		
<i>Total</i>	<i>146</i>	<i>230.0</i>	<i>1.594</i>			
End of the epidemics						
Among plots	1	2.8	0.014	0.80	0.074	0.008
Within plots	183	312.6	1.708	99.20		
<i>Total</i>	<i>184</i>	<i>315.3</i>	<i>1.722</i>			

(P05, P13; Table S3B). In all cases, the variation within populations accounted for most of the molecular variance (89.2-100%). Variation among ascospore and pycnidiospore populations were low and non significant for D+ plots (-0.4-3.4%; $0.116 < P < 0.659$; $-0.004 < F_{ST} < 0.033$) but also for D₀ plots (0.57% and 10.1%; $0.118 < P < 0.279$; $0.006 < F_{ST} < 0.101$). Such a lack of difference in the genetic structure of the populations does not allow in D+ plots to conclude, overall, about the origin - local vs. distant - of primary inoculum for each plot (i.e. originating from resident vs. immigrant populations).

Evolution of population diversity during the three successive cultural seasons

Genetic variability within each of the 26 *Z. tritici* populations (Figure 1) was estimated first by the genic non biased heterozygosity diversity, H_E (Figure 5A). H_E values were in the same order of magnitude for all populations and varied between 0.38 and 0.58, with a mean at 0.46, 0.44 and 0.42 for the pycnidiospores populations from D+, D₀ and ascospore populations, respectively. At last, no significant difference was found between the evolution of populations from the D+ and D₀ plots, according to *t*-test for paired populations ($P = 0.471$).

The mean number of alleles per locus (allele richness A_r) ranged between 2 and 6.27 (mean 4.45, 3.67 and 4.06 for the D+, D₀, and ascospores populations, respectively) for the three cultural seasons (Figure 5B). The highest A_r values were found for season 2011-2012, which is also the season with the highest number of strains collected. Allele richness was always equivalent or greater in the D+ plots compared to the D₀ ones, with one exception found for the second intermediate period of season 2011-2012, not consistent with difference in population size. No significant difference was observed between the D+ and D₀ populations over time ($P = 0.084$).

Occurrence of sexual reproduction in *Z. tritici* field populations

The two mating-types Mat1-1 and Mat1-2 were found in all populations (Figure 6A), excepted for P13, a D₀ pycnidiospore population composed of only five individuals of Mat1-2 type. The mating-type ratio Mat1-1/Mat1-2 calculated for all the populations, including only isolates with complete mating type (703 among 800 isolates), was 0.95. Results of χ^2 tests showed that the frequency of the two mating types was not significantly different from the expected 1:1 ratio ($P = 0.126$).

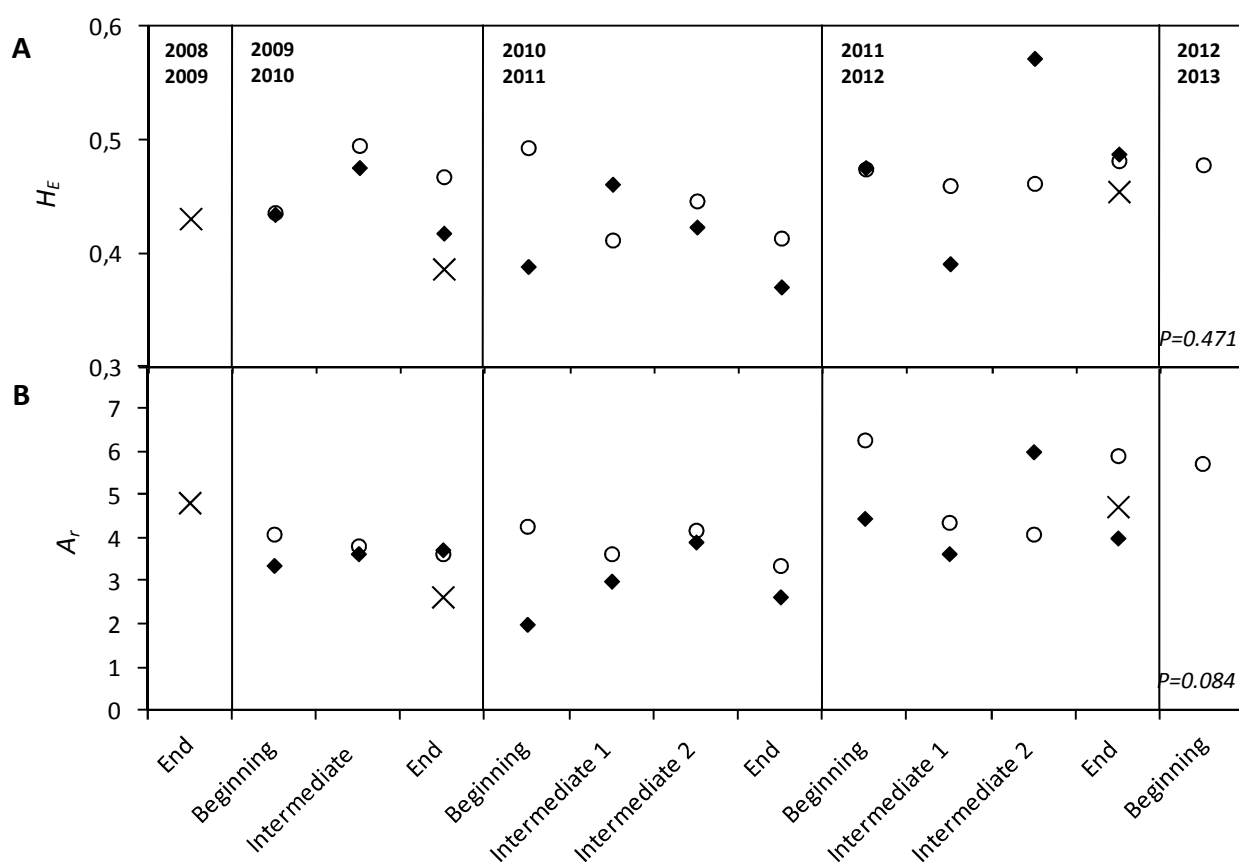


Figure 5 - Temporal evolution of the diversity of 26 *Z. tritici* populations (800 isolates; see Figure 1) collected in wheat plots with (D+) or without (D₀) debris during three cultural seasons (2009-2010, 2010-2011 and 2011-2012). A - Observed heterozygosity (H_E). B - Allele richness (A_r , mean number of alleles per locus). White circles: pycnidiospore populations from D+ plots; black diamonds: pycnidiospore populations from D₀ plots; crosses: ascospore populations.

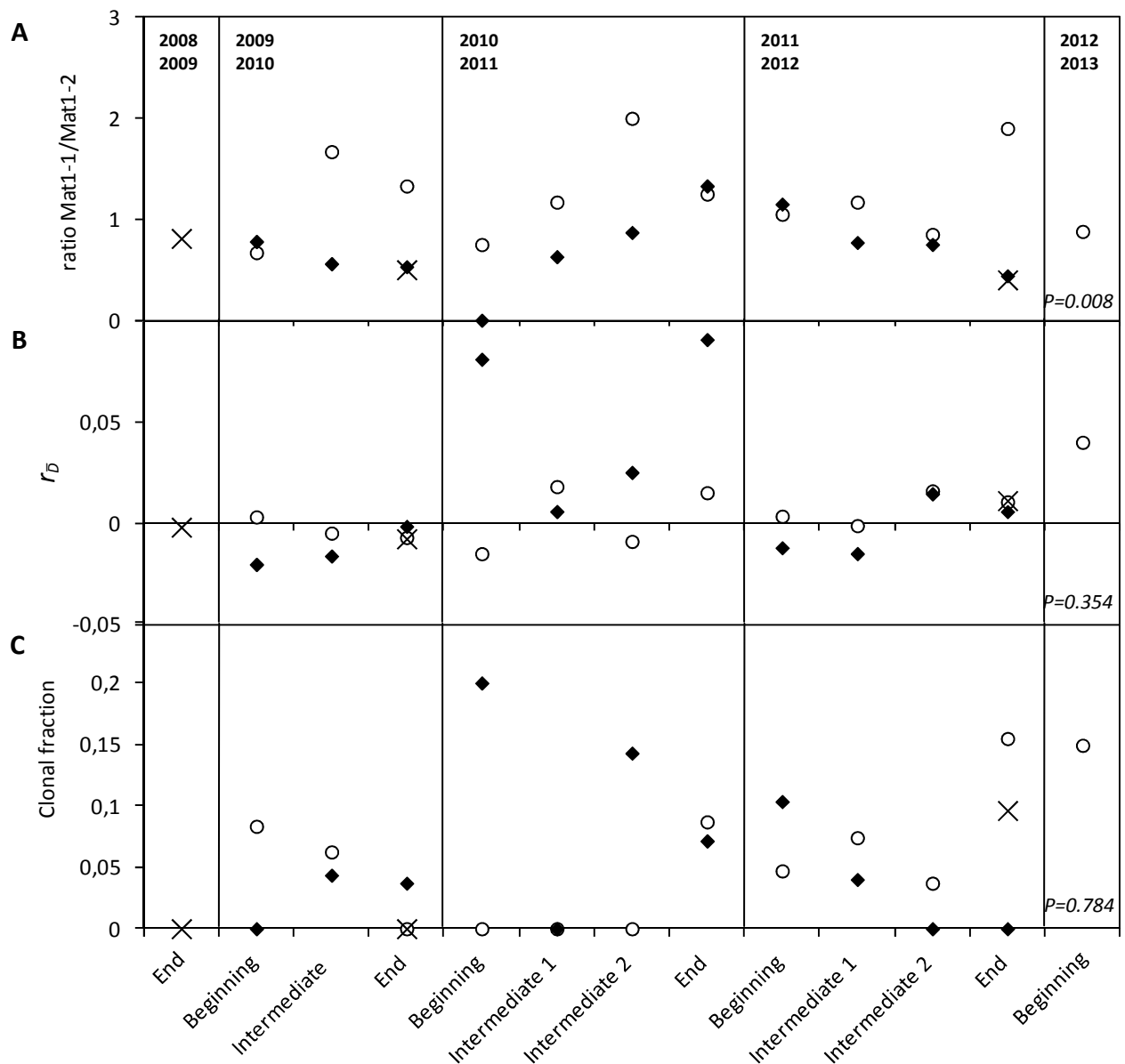


Figure 6 - Evidence of sexual reproduction of 26 *Z. tritici* populations (800 isolates; see Figure 1) collected in wheat plots with (D⁺) or without (D₀) debris during three cultural seasons (2009-2010, 2010-2011 and 2011-2012). A - Temporal evolution of the mating-type ratio (Mat1-1/Mat1-2) in pycnidiospores and ascospores populations. B - Temporal evolution of the multilocus linkage disequilibrium estimated as the r_D index. C - Temporal evolution of the clonal fraction (1-G/N) in the same plots. Marks indicate the origin of the populations: white circles: pycnidiospores populations from D⁺ plots; black diamonds: pycnidiospore populations from D₀ plots; crosses: ascospore population.

Difference in mating-type ratio was then assessed between plots. The mean ratio was 1.22 in the D+ pycnidiospore populations and 0.71 in the D₀ pycnidiospore populations (Figure 6A). Results of χ^2 tests showed that the frequencies of the two mating types within each plot were not significantly different from 1:1 ($P = 0.407$ for D+; $P = 0.746$ for D₀). The results of the t -test for paired samples showed that the difference in ratio between the two plots over time was however significant ($P = 0.008$). Finally, difference in mating-type ratio was assessed between epidemic periods. Results of χ^2 tests showed that the frequency of the two mating types in pycnidiospore populations collected at the epidemic periods "end" were significantly different from 1:1 (mean ratio = 1.26; $P = 0.047$), but not at the epidemic periods "beginning" ($P = 0.687$).

Linkage disequilibrium was always low (close to full panmixia), with r_D ranging from negative values to a maximum of 0.09 (P16), with a mean value of 0.006, 0.015, and 0.001 for the D+, D₀, and ascospore populations, respectively (Figure 6B). Again, no significant differences between the D+ and D₀ plots were found ($P = 0.354$), despite r_D was greater at the beginning and at the end epidemic periods of the 2010-2011 season in D₀ plot.

The clonal fraction was very low over the three cultural seasons (Figure 6C): in average 0.06 for pycnidiospore populations from both D+ and D₀ plots, and 0.03 for ascospore population, with values ranging from a minimum of 0 (no clone found in the population) to a maximum of 0.2 (only two clones in the 5-isolate population P13). No significant difference in the clonal fraction between the two plots was recorded ($P = 0.784$).

Discussion

In this study, we aimed to identify neutral structure at a plot scale and over three cultural seasons, first considering a without *a priori* approach for individual assignment and testing three putative structuring factors: time period, origin of isolates and type of spores. We found no relevant neutral population structure, due to any of these factors. There were especially no differences in the genetic structure of populations collected early and late in the same season, or between collections made in the same plot over a three year period. This is consistent with several previous results which showed that the genetic structure of *Z. tritici* populations was stable in time, over 3-year (Chen et al., 1994) and 6-year periods (Zhan et al., 2001), and in space, from spatial scales ranged from meters to thousands of kilometers (Linde et al., 2002 ;

Schnieder et al., 2001). Accordingly, the 800 isolates collected locally constituted a single, highly diverse entity that remained stable along all the epidemic stages, whatever the demography of the population. These results mean that neither genetic drift nor possible bottlenecks (for instance caused by unfavorable climatic conditions or prophylaxis) may sufficiently operate to differentiate sub-populations distinguishable by our neutral markers set. This large, unstructured population did not appear to be affected by the origin of the inoculum colonizing the plots each year, and especially not by the introduction of airborne inoculum. Airborne inoculum might have been constituted of local genotypes resulting from sexual reproduction occurring in the studied plots, but also, at least partly, of genotypes of distant origin, considering the long-distance dispersal capacities of *Z. tritici* ascospores. Indeed, the wheat field without debris (D₀) was recolonized in each year by inoculum of unknown distant origin, while the wheat field with debris (D₊) was mainly recolonized by local inoculum, as showed by previous results (Suffert & Sèche, 2011; Morais et al, unpublished data) obtained in the same experimental area. The lack of genetic structure, which means that ascospores and pycnidiospores share neutral alleles at similar frequencies, is not surprising: firstly, pycnidiospores populations derive from ascospores populations after a limited number of clonal reproduction cycles (maximum five, depending to the epidemic period; Agrios, 2005); secondly, SSRs were likely not "filtered" (i.e. selected) by the wheat cultivar, which also confirms the neutrality of the markers used in this study.

Analysis of molecular variance (AMOVA) of populations defined *a priori* based on our sampling scheme (Figure 1), revealed that sampling time, especially the epidemic period, could be a slight (< 2% of molecular variance) but significant determinant of the differentiation of pycnidiospore populations, in the two studied plots. Similarly, the origin of isolates, contrasted through different debris management in the two plots, explained a slight but significant part (< 2.5%) of molecular variance during the growing season (intermediate periods 1 and 2, i.e. between February and May), when *Z. tritici* populations are supposed to undergo demographical expansion through asexual reproduction. At last, we found that molecular variance was similar among ascospore and pycnidiospore populations collected at the end of the cropping season, with one exception: the differentiation between the pycnidiospore population from the plot without debris at the end of epidemic period (P27; season 2011-2012) and the ascospore population from the plot with debris collected at the beginning of the next season (P28; 2012-2013) was low but significant. Because *Z. tritici* are known to be highly diversified at large and small spatial scales, the overall lack of genetic

variation for neutral markers between populations collected in D+ and D₀, at the same epidemic period, was not surprising. From a demographic point of view, it could be hypothesized that a limited local inoculum, potentially less diversified than a large airborne inoculum, has a greater quantitative impact on early epidemic stages than distant inoculum. From a genetic point of view, it could be also hypothesized that immigration has a qualitative impact with opposite consequences: distant airborne inoculum could increase the overall genetic diversity by adding new alleles in a local population. The resulting demogenetic impact of such contradictory effects probably varies with the year and with the local vs. distant inoculum pressure. Indeed, our results showed that some differences can be established sporadically: the peculiar case P27-P28 suggests a strong but transient contribution of local ascospore population, consistently with results of a quantitative epidemiological approach (Suffert & Satche, 2011).

Genetic diversity was high to very high in all populations and stable over the three cultural seasons. PCA (Figure 3) and pairwise F_{ST} index (Table S2) confirmed however that some populations appeared slightly more differentiated than other. Such populations were mostly collected during the 2011-2012 cultural season (P18, P19, P25, and P27). It is likely that the reproductive trajectory of the aforementioned populations of the season 2011-2012, collected in D₀ as in D+, was characterized by a sexual recombination during spring lower than during the two previous cultural seasons. This hypothesis is consistent with the local epidemiological context: at the end of the cultural season 2010-2011, the disease severity was very low: lesions were absent from flag leaf at mid-June 2011, while they covered 2-5% in 2010 and 15-20% in 2012. As expected (Cowger et al., 2002), the ascospore production from debris derived from this crop at the beginning of the 2011-2012 cultural season was undetectable using the methodology described in Suffert & Satche (2011): no ascospore were collected from debris at the end of autumn 2011, against 1-5 ascospores.g⁻¹ and 5-20 ascospores.g⁻¹ at the end of autumn 2010 and 2012, respectively. Therefore, it is likely that the amount of airborne ascospores available during spring 2012, produced from debris in D+ plots and in other distant fields, was negligible or almost zero. The proportion of secondary infections induced by ascospores released either from old, still infectious debris located in distant plots or from infected wheat plants located in the same plot (Clinckemaillie *et al.*, 2010; Duvivier *et al.*, 2013), were probably very low or null in comparison with the two previous cultural seasons.

The order of magnitude of the expected heterozygosity (H_E), of the allele richness (A_r) and of the proportion of multiple haplotypes was consistent, although somewhat smaller, with values obtained with SSR markers at a larger geographic scales in Tunisian, French or Canadian populations (Linde et al., 2002; Razavi & Hughes, 2004; El Chartouni et al., 2011; Boukef et al., 2012). Ascospore and pycnidiospore populations could not be distinguished for their diversity, whereas higher levels of genotypic diversity could have been expected after sexual recombination. Similarly, no significant difference was found in diversity evolution between the two plots, meaning that the greater amount of local primary inoculum produced in the stubble plots had no or little consequence on diversity.

The frequency of the two mating types was not significantly different from the expected 1:1 ratio, consistently with previous results (Zhan et al., 2002; Siah et al., 2010). The presence of both idiomorphs Mat 1-1 and Mat 1-2 in all populations, although in variable proportions, indicate that sexual reproduction could occur all the year round, even if specific climatic conditions and host tissue physiological status are required. The χ^2 test on mating-type frequencies of Mat1-1 at the beginning and at the end of epidemic periods (not significantly different from 50%, and significantly higher, respectively) illustrated a slightly increase in the frequency of the Mat 1-1 isolates during a cultural season. This result may be related to the greater pathogenicity of Mat 1-1 isolates than Mat 1-2 isolates (Zhan et al., 2007), which should be explored with *in planta* experiments with our isolates.

Linkage disequilibrium was always very low over time and close to full panmixia, independently of the origin of the primary inoculum. Similarly, the clonal fraction was always low, whatever the sampling time and the origin of the inoculum. As an example, the cultural season 2009-2010 was distinguished by intense ascospore production in the stubble plot, comparatively to the two other studies seasons (unpublished data), which did not have much impact on the evolution of the diversity indexes. Altogether, these results are consistent with the recombination due to sexual reproduction over a long period of the year and again, are in the same order of magnitude of what is already known for this fungal species at larger geographical scale. This suggests that local stubble management, while decreasing the local production of ascospores and then modifying the amount of primary inoculum, may be compensated by intense migration.

In conclusion, local *Z. tritici* populations showed a high degree of genetic uniformity from year to year, from epidemic period to epidemic period, and from plot to plot,

consistently with results obtained by Chen et al. (1994), Zhan & McDonald (2002) and El Chartouni et al. (2012). Demogenetic analysis of local populations suspected to result from different evolution trajectories through three successive sequences of intra- and inter-epidemic periods, allowed us to underscore some sporadic effects of the origin of inoculum, depending of the year and the epidemic period. Such effects were slight but statistically significant. The overall genetic stability measured at a small spatiotemporal scale is definitively not contradictory with signs of differentiation. In the case of a cyclic parthenogen whose dual sexual-asexual reproductive modes can expressed concomitantly within the same year (Dilmaghani et al., 2013; Castel et al., 2014), the genetic diversity, for traits subjected to selective pressure that vary within the cultural season, is expected to be lower in populations having mainly a clonal reproduction trajectory than in a population resulting of sexual reproduction. Such diversity in neutral markers, if it exists, is probably higher because they are theoretically not impacted by selective pressure. Even so, in our study, the slightly lower differentiation between pairs of ascospore populations and between ascospore and pycnidiospore populations than between pairs of pycnidiospore populations was sufficient to conclude that the role of ascospores as inoculum vary with the cultural season and the epidemic period. Analogous hypotheses about the respective contribution of sexual/asexual primary/secondary inoculum were tested by Cortesi & Milgroom (2001) and Shah et al. (2001) looking at results from spatial patterns and genetic diversity. Such studies, still uncommon at a small spatiotemporal scale, exemplifies how population genetics can be used directly to address epidemiological questions essential for sound disease management (Milgroom & Peever, 2003). Previous studies showed that *Z. tritici* inoculum was quantitatively not limiting in Western Europe conditions (Suffert & Satche, 2011). Our findings complete these results and suggest that, the years when the amount of inoculum is very low, the interepidemic period can induce slight neutral genetic variation within local populations, however detectable by neutral marker analysis. Such effect would be likely higher with genes subject to selective pressure (fungicide or cultivar resistance gene). Overall, we confirmed that the interepidemic period can have an impact, at least small and occasional, on the neutral genetic structure, even for a fungal population known to be highly diverse and strongly conditioned by sexual recombination.

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Table S1 - Results of the clustering analysis performed with STRUCTURE for isolates collected during three cultural seasons (2009-2010, 2010-2011 and 2011-2012) and genotyped with 11 SSR markers (Gautier et al., 2014).

All runs were replicated 10 times (burn in period = 500000 and MCMC iterations = 1000000), for each cultural season and overall. Bold values represent the optimal number of clusters K (Evanno et al., 2005); clustering corresponding to underlined values is presented in Figure 2.

A. Effect on epidemic period with pycnidiospore populations from D+ plot (460 isolates)

K	2009-2010		2010-2011		2011-2012		Overall	
	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K
1	—	—	—	—	—	—	—	—
2	6.810	1.981	16.780	14.376	170.100	20.254	7.310	2.277
3	17.170	2.310	1.850	0.526	1226.580	13.177	83.810	3.797
4	2.200	0.144	6.720	0.573	870.310	2.938	138.920	2.860
5	6.270	0.853	33.250	1.682	1516.770	1.965	115.270	1.050
6	7.040	0.551	5.740	0.314	803.630	2.334	417.540	1.169
7	4.300	0.523	20.250	1.144	162.970	0.864	<u>3314.540</u>	<u>13.881</u>
8	1.290	0.146	18.130	0.662	78.210	0.705	860.940	0.497
9	2.120	0.223	0.270	0.012	39.590	0.567	3841.650	1.941
10	—	—	—	—	—	—	—	—

B. Effect of origin of isolates (D+ and D₀ plots) with pycnidiospore populations (681 isolates)

K	2009-2010		2010-2011		2011-2012		Overall	
	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K	$ \ln''(K) $	Delta K
1	—	—	—	—	—	—	—	—
2	4.920	2.128	18.490	17.034	202.800	27.063	<u>1396.743</u>	<u>17.451</u>
3	15.600	3.907	7.950	1.716	836.650	16.871	1782.690	6.325
4	7.570	0.820	6.020	0.515	445.660	1.656	779.260	1.771
5	4.740	0.317	2.830	0.166	139.360	0.508	311.320	0.847
6	23.550	1.183	26.720	1.501	1100.370	0.913	1203.560	3.404
7	56.210	1.291	26.470	1.783	368.040	0.833	476.760	3.336
8	35.720	1.227	26.980	0.814	258.460	0.170	363.870	0.434
9	21.550	0.790	20.320	1.408	99.900	0.071	907.120	1.504
10	—	—	—	—	—	—	—	—

C. Effect of type of spores (ascospore and pycnidiospore) with populations from D+ and D₀ plots (800 isolates).

<i>K</i>	Overall	
	Ln ⁿ (<i>K</i>)	Delta <i>K</i>
1	—	—
2	133.443	15.668
3	<u>1031.870</u>	<u>18.864</u>
4	138.800	0.497
5	727.010	2.641
6	168.030	0.268
7	1155.840	2.663
8	1298.330	2.022
9	654.750	1.076
10	—	—

Table S2 - Pairwise F_{ST} between the 26 *Z. tritici* populations (see Figure 1) collected in Thiverval-Grignon (France), classified according to type of spore (pyncnidiospore, ascospore), cultural season (2009-2010, 2010-2011 and 2011-2012), epidemic period (beginning, intermediate 1, intermediate 2, end) and origin (D+ plot, D₀ plot). Bold values indicate F_{ST} with $P < 0.05$ (significant); $F_{ST} > 0.016$ (the F_{ST} overall mean) are highlighted in yellow; the rank of the populations with the four higher marginal F_{ST} means is indicated in fuchsia frame.

Type of spores	pyncnidiospores												ascospores							
	Cultural season			2009-10				2010-11				2011-12				2012-13	2008-09	2009-10		
	Epidemic period	Beginning		Intermediate		End		Beginning		Intermediate 1		Intermediate 2		End		Beginning	End			
D+		D ₀	D+	D ₀	D+	D ₀	D+	D ₀	D+	D ₀	D+	D ₀	D+	D ₀	D+	D ₀				
pyncnidiospores	2009-10	Beginning	D ₀	P05	0.002													P02	0.014	
		Intermediate	D+	P03	0.017	0.009													P06	0.011
		End	D+	P04	0.004	-0.011	0.022	0.016											P03	0.013
	2010-11	Beginning	D ₀	P07	0.012	0.025	0.021	0.001	0.008										P06	0.017
		Intermediate 1	D+	P09	0.008	-0.012	0.018	0.020	0.006	0.012									P07	0.010
		Intermediate 2	D+	P10	0.042	0.020	-0.020	-0.007	0.034	0.018	0.007								P09	0.015
	2011-12	Beginning	D+	P11	-0.006	0.023	0.013	0.020	0.017	0.006	0.003	0.051							P13	0.025
		Intermediate 1	D+	P12	0.008	-0.020	0.001	-0.004	-0.010	-0.008	0.005	0.027	-0.006	0.026	0.013				P14	0.012
		End	D+	P16	0.060	0.061	0.044	0.019	0.082	0.037	0.041	0.078	0.018	0.039	0.027	0.021	0.043		P12	0.005
	2012-13	Beginning	D+	P17	0.005	0.002	0.000	0.009	0.008	0.002	0.005	0.029	0.005	0.011	-0.007	0.015	0.000	0.033	P17	0.010
		Intermediate 1	D+	P18	0.023	0.030	0.010	0.033	0.028	0.014	0.037	0.016	0.010	0.011	0.011	0.036	0.024	0.031	P23	0.005
		Intermediate 2	D+	P19	0.034	0.026	0.041	0.035	0.037	0.008	0.022	-0.013	0.029	-0.008	0.018	0.037	0.033	0.049	P18	0.020
	ascospores	Beginning	D+	P21	0.016	-0.005	0.008	0.011	0.004	0.005	0.018	0.026	0.003	0.015	0.018	0.000	-0.011	0.035	P25	0.028
		End	D+	P22	0.026	0.045	0.027	0.057	0.040	0.016	0.034	0.017	0.042	0.000	0.028	0.040	0.032	0.065	P29	0.008
		Beginning	D+	P29	-0.001	0.018	-0.003	0.006	0.011	-0.002	0.018	0.011	-0.004	0.002	-0.001	0.000	0.006	0.022	0.004	-0.004
	ascospores	Beginning	D+	P01	-0.002	-0.001	0.023	0.025	0.005	0.013	0.017	0.064	0.006	0.013	0.005	0.007	-0.003	0.048	P01	0.015
End		D+	P08	0.023	0.000	0.012	0.009	0.011	-0.001	0.014	0.069	-0.023	0.011	0.015	-0.029	-0.019	-0.008	P08	0.007	
Beginning		D+	P28	0.001	0.001	0.011	0.011	-0.012	-0.010	0.010	0.018	0.004	-0.001	0.000	-0.004	-0.002	0.038	P28	0.004	

Table S3 - Analyses of molecular variance (AMOVA) performed to test, at relevant epidemic periods (end/beginning), the effect of the type of spores on the genetic structure of the pycnidiospore and ascospore populations collected in Thiverval-Grignon, France (see Figure 1). A - Each ascospore population collected from wheat debris at the beginning of epidemics (P08, P28) was compared with the pycnidiospore population collected at the end of the previous cultural season in D+ plots (P04, P21, respectively), and with the pycnidiospore population collected in D₀ plot (P07, P27, respectively). B - Each ascospore population collected from wheat debris at the beginning of epidemics (P01, P08, P28) was compared with the pycnidiospore population collected during the same period in D+ plots (P02, P09, P29, respectively), and with the pycnidiospore population collected in D₀ plots (P05, P13).

A	Df	Sum of squares	Variance components	% of variation	<i>P</i> -value	<i>F_{ST}</i>
P08 (ascospore) vs P04 (D+)						
Among populations	1	2.899	0.031	1.20	0.296	0.012
Within populations	28	71.034	2.537	98.80		
<i>Total</i>	<i>29</i>	<i>73.933</i>	<i>2.568</i>			
P08 (ascospore) vs P07 (D₀)						
Among populations	1	1.418	-0.034	-1.90	0.721	-0.019
Within populations	33	60.782	1.841	101.90		
<i>Total</i>	<i>34</i>	<i>62.200</i>	<i>1.808</i>			
P28 (ascospore) vs P21 (D+)						
Among populations	1	1.486	-0.005	-0.29	0.664	-0.003
Within populations	134	245.095	1.829	100.29		
<i>Total</i>	<i>135</i>	<i>246.581</i>	<i>1.824</i>			
P28 (ascospore) vs P27 (D₀)						
Among populations	1	2.732	0.052	3.35	0.045	0.033
Within populations	65	98.283	1.512	96.65		
<i>Total</i>	<i>66</i>	<i>101.015</i>	<i>1.564</i>			

B	Df	Sum of squares	Variance components	% of variation	<i>P</i> -value	<i>F_{ST}</i>
P01 (ascospore) vs P02 (D+)						
Among populations	1	1.9	-0.009	-0.40	0.638	-0.004
Within populations	89	206.2	2.317	100.40		
<i>Total</i>	<i>90</i>	<i>208.1</i>	<i>2.308</i>			
P01 (ascospore) vs P05 (D₀)						
Among populations	1	2.0	0.010	0.57	0.279	0.006
Within populations	69	121.2	1.756	99.43		
<i>Total</i>	<i>70</i>	<i>123.2</i>	<i>1.766</i>			
P08 (ascospore) vs P09 (D+)						
Among populations	1	2.2	0.054	3.35	0.166	0.033
Within populations	30	46.8	1.560	96.65		
<i>Total</i>	<i>31</i>	<i>49.0</i>	<i>1.614</i>			
P08 (ascospore) vs P13 (D₀)						
Among populations	1	2.0	0.135	10.11	0.118	0.101
Within populations	11	13.2	1.200	89.89		
<i>Total</i>	<i>12</i>	<i>15.2</i>	<i>1.335</i>			
P28 (ascospore) vs P29 (D+)						
Among populations	1	0.8	-0.004	-0.42	0.659	-0.004
Within populations	117	129.0	1.103	100.42		
<i>Total</i>	<i>118</i>	<i>129.8</i>	<i>1.098</i>			

Inferring the origin of primary inoculum of *Zymoseptoria tritici* from local host adaptation of resident and immigrant populations *

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Abstract

We investigated whether the main origin of primary inoculum of a fungal plant pathogen (*Zymoseptoria tritici*) can be inferred from the adaptation to its host. We compared the aggressiveness of two local populations, the first considered as resident (12 isolates collected from debris in a 3-year wheat cv. Soissons monoculture plot) and the second considered as immigrant (12 isolates collected from leaf lesions in a 300 m apart wheat cv. Soissons plot containing no debris, exposed to inoculum pool of distant origin), with the aggressiveness of a third local pathogen population (12 isolates collected from early leaf lesions in the monoculture plot) the origin of which we wanted to determine. Latent period and sporulating area were assessed on adult wheat plants in a greenhouse, on the sympatric host (cv. Soissons) and on an allopatric host (cv. Apache), both widely cultivated in the agricultural basin. Firstly, we underscored a local host adaptation in the *Z. tritici*-wheat system at a fine spatiotemporal scale, based on the fact that the resident population became less adapted to the allopatric host (longer latent period) after a 3-year monoculture. Secondly, we showed that the aggressiveness profile of the third pathogen population was closer to that of the resident population than of the immigrant. We concluded that the early lesions that appeared in the monoculture plot were mostly caused by within-field primary inoculum. We therefore established that the origin of *Z. tritici* primary inoculum for wheat after wheat crop was more local than distant.

Keywords

aggressiveness; allopatry vs. sympatry; host adaptation; inoculum source; *Mycosphaerella graminicola*; wheat

Introduction

Primary inoculum can be defined as the pathogen unit (most often, a spore) at the origin of a disease outbreak observed in a given host unit (for instance, a field plot). At the end of a cropping season, the pathogen locally faces unfavorable environmental conditions and the lack of crop host through different mechanisms, such as infection of an alternate (Roelfs, 1985; Anikster *et al.*, 1997; Jin *et al.*, 2010) or accessory host (Holmes & Dennis, 1985; Suffert *et al.*, 2011), or survival on crop debris (Landschoot *et al.*, 2011; Suffert & Sache, 2011). When local conditions do not permit survival, re-colonization of the crop unit must be achieved every year by pathogen units spread over long distance from remote sources of inoculum, located in areas conducive to survival (Strange, 2003; Wang *et al.*, 2010). The origin of primary inoculum remains a matter of speculation in several plant diseases, because of the lack of biological knowledge on non-epidemic (survival) stages of epidemics; of the potentially infinite number of sources scattered over huge areas; and of the difficulty to record the small amounts of inoculum theoretically able to initiate disease. Accordingly, accurately tracking back the origin of a disease outbreak has been for decades a challenge to epidemiologists, who have to find a needle in a haystack.

Direct evidence of the presence of inoculum can be obtained by trapping and quantifying airborne spores during distinct epidemic periods in given plant unit (Calderon *et al.*, 2002; Fraaije *et al.*, 2005; Kaczmarek *et al.*, 2012; Karolewski *et al.*, 2012). Such methods still have technical limitations, which often prevents the detection of the very low amounts of spores thought to initiate the first infections, and are, therefore, more suitable to identify inoculum peaks over the epidemic season (Carisse *et al.*, 2009; Duvivier *et al.*, 2013). Indirect evidence of the origin of inoculum could rather be searched by comparing the genetic structure or by assessing the fitness traits of pathogen populations confronted to a local host. Immigrant pathogen populations can be expected to exhibit a competitive disadvantage when compared with resident, locally adapted populations.

Local adaptation of a pathogen population is thought to be a consequence of the specialization of the local population on the most common local host genotypes. Pathogens are considered to be locally adapted if they perform better on sympatric than on allopatric hosts (Gandon & Van Zandt, 1998; Kaltz & Shykoff, 1998). Symmetrically, pathogens are considered to be locally maladapted if they perform lower on sympatric than on allopatric

hosts (Kaltz *et al.*, 1999). Pathogen strains co-infecting a host may originate from distinct pathogen populations and possess different degrees of genetic relatedness. Some pathogen strains may be locally adapted residents that have been selected through many generations of reproduction, whereas other may be newly arrived immigrants that were adapted to a different environment. On average, immigrants are expected to be at a competitive disadvantage compared with residents because they have not been selected for adaptation to the local environment (Giraud, 2006; Gladieux *et al.*, 2011) and therefore cannot maintain their population size because of lower basic reproduction and/or transmission rates (Zhan *et al.*, 2002; Laine, 2008).

Local adaptation of plant pathogens can be assessed using derived fitness estimates (Laine, 2008), here aggressiveness components, such as latent period, spore production rate, lesion size, infection efficiency or toxin production (Lannou, 2012). Aggressiveness can be defined as a quantitative variation in pathogenicity, the ability of pathogens to infect and grow on susceptible hosts (Zhan *et al.*, 2013b); its components were assessed in several pathogens, using various experimental techniques (Pariaud *et al.*, 2009a; Suffert *et al.*, 2013; Azzimonti *et al.*, 2013).

Epidemics of *Septoria tritici* blotch of wheat, caused by the fungus *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*, anamorph *Septoria tritici*; Quaedvlieg *et al.*, 2011) are generally believed to be initiated by ascospores wind-dispersed from infected wheat debris (Shaw et Royle, 1989), although other sources of inoculum cannot be firmly excluded (Suffert *et al.*, 2011). Whether primary inoculum originates mostly from local or remote sources is unknown yet.

In this study, we investigated whether the origin of primary inoculum of *Z. tritici* can be inferred from host adaptation of different local pathogen populations. To this aim, we compared the aggressiveness of two local populations of the pathogen, the one considered as resident and the other considered as immigrant, with the aggressiveness of a third local population having an unknown origin. The sporulating area and the latent period, measured under greenhouse conditions, were previously shown to be relevant for the assessment of aggressiveness in a panel of isolates of *Z. tritici* (Suffert *et al.*, 2013). Three questions were addressed: i) Can we detect differences in aggressiveness between the resident and immigrant populations, and, more precisely, a differential competitive advantage between them? ii) Can these differences be interpreted as evidence of local host adaptation, especially to sympatric

or allopatric hosts? iii) Can the local vs. distant origin of the third population be inferred from the comparison of its aggressiveness profile to those of the resident and immigrant populations?

Materials and methods

Sampling of pathogen populations

Two plots were sown 300 m apart with wheat cv. Soissons (moderately susceptible to *Septoria tritici* blotch; rated 5 on a 1-10 scale of decreasing susceptibility, Arvalis Institut du Végétal-CTPS) in autumn 2009 at the Grignon experimental station (France, 48°51'N, 1°58'E). Soil and weather characteristics, as well as cropping practices, have been described elsewhere (Suffert & Sèche, 2011). The first plot (D+) had been grown with wheat cv. Soissons by direct drilling, without fungicide application, since 2007 (Figure 1a). Wheat debris were not removed in 2009 after harvest; naturally infected with *Z. tritici*, the debris were expected to act in autumn as a local source of primary inoculum. The second plot (D₀) did not contain any wheat debris since the plot had been grown the previous season with oilseed rape (Figure 1b).

Three populations each made of 12 isolates were sampled from the wheat plots (Figure 2; Table S1). The first population (P1), hereafter named "resident population", was considered as a sample of the local inoculum pool. It was obtained from debris collected on 23 October 2009 in plot D+. Debris were cut into 1-cm long pieces kept at 18°C for 24 h. A subset of these pieces (20 g) was evenly spread on wet filter paper enclosed in a moist box. Petri dishes (90 mm in diameter) filled with PDA medium (potato dextrose agar, 39 g.L⁻¹) were placed upside down 1 cm above the debris pieces (Figure 1c). The boxes were placed for 6 h at 18°C in the dark to trigger ascospore ejection. Thereafter the Petri dishes were closed and kept in the same conditions to promote yeast-like growth of the fungus (Suffert & Sèche, 2011). Five days after ascospore ejection, *Z. tritici* colonies were isolated from the growing mycelium and purified by monospore culture to obtain 12 isolates.

The second population (P2) was obtained from infected leaves sampled from 24 November to 8 December 2009 in plot D₀. This population, hereafter named "immigrant population", was considered as a sample of an inoculum pool of distant origin, that is, built-up by ascospores ejected from distant debris. Since no infected wheat debris was present in the neighbourhood

of plot D₀, infections were exclusively caused by wind-dispersed, distant ascospores, of unknown origin.

The third population (P3) was obtained from infected leaves sampled on 24 November 2009 in plot D+. This population, hereafter named "tested population", was assumed to have a mixed origin, that is, built-up by ascospores ejected from debris of either local (present on the soil of plot D+) or distant origin, in an unknown proportion.

To obtain the isolates composing populations P2 and P3, leaves collected from plots D+ and D₀, respectively, were placed on wet filter paper enclosed in a moist box kept for 24 h at 18°C in the dark to promote cirrus exudation (Suffert & Sache, 2011). A single cirrus from a single pycnidium per lesion (Figure 1d) was transferred to a Petri dish filled with PDA and then streaked across the agar surface with a sterile glass rod to separate individual spores. Petri dishes were incubated at 18°C in the dark to promote yeast-like growth of the fungus. *Z. tritici* colonies were isolated from the growing mycelium and purified by monospore culture to obtain 2 × 12 isolates.

The 36 isolates used in this study have distinct genotypes, as evidenced by distinct SSR neutral markers (unpublished data; Gautier *et al.*, 2014). They were stored in a sterile glycerol-water (1:1) solution kept at -80°C.

Plant material

Wheat adult plants of cv. Apache and Soissons were grown in a greenhouse for subsequent inoculation with the aforementioned fungal isolates. Like Soissons, Apache is moderately susceptible to septoria tritici blotch (rated 5 on a 1-10 scale of decreasing susceptibility, Arvalis Institut du Végétal-CTPS). Soissons and Apache have been commercially grown around the experimental site for several years. Here we consider Soissons as the sympatric host of the three pathogen populations, that is, the cultivar from which the fungal isolates were collected (Kaltz & Shykoff, 1998; Laine, 2007), while we consider Apache as an allopatric host.

Wheat seeds were sown on 7 December 2011 in Jiffy peat pots (Jiffypot®, Jiffy France SARL). Seedlings were vernalized for seven weeks at 8°C with a 10 h light period and a 14 h dark period. Afterwards, seedlings were brought back to the greenhouse and left to acclimatize for one week. Plants were then transplanted into 1.1 L pots filled with 1 L of

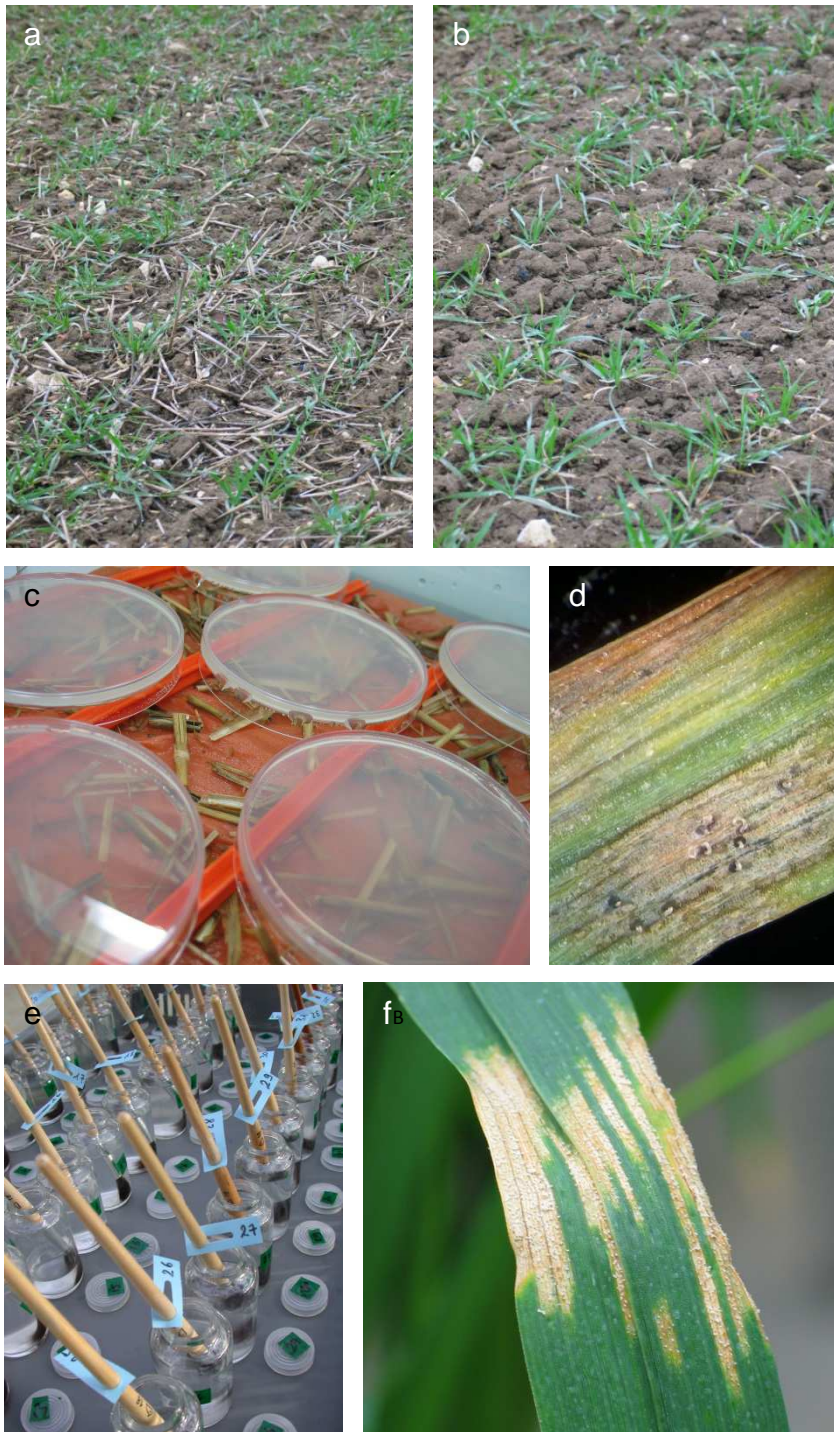


Figure 1 - (a) Wheat plants cv. Soissons in the wheat monoculture plot with debris (D+) and (b) in the plot without debris (D₀) at the early stages of septoria tritici blotch epidemic. (c) Petri dishes containing PDA medium placed upside down above wheat debris fragments collected in plot D+ in order to collect ascospores. (d) *Zymoseptoria tritici* pycnidia and cirri on wheat plants collected in plot D+ and D₀. (e) Inoculum suspensions. (f) Disease lesions on flag leaf of wheat cv. Apache in the greenhouse experiment.

commercial compost (Klasmann® Substrate 4; Klasmann-Deilmann France SARL) and fertilized with 5 g Osmocote® Exact (16N-11P-11K + 3MgO + TE). Plants were sprayed with Spiroxamine (Aquarelle SF at 2 mL.L⁻¹; Bayer CropScience, France) against powdery mildew (*Blumeria graminis* f.sp. *tritici*). In addition, plants were fertilized with a commercial nutrient solution (Hydrokani C2®, Hydro Agri Spécialités) at a 1:100 dilution rate poured in the pot saucers five and seven weeks after seedling transplantation. During plant growth, natural daylight was supplemented with 400 W sodium lamps to obtain 9h of dark and 15h of light, the greenhouse temperature being kept under 20°C during the light period and above 12°C during the dark period. Three stems per plant were kept. The air temperature in the greenhouse compartment was recorded every 15 minutes using a ventilated sensor (Greenhouse Humidity and Temperature Sensor type 224.401; RAM, Germany). The thermal time *t*, expressed in degree-days, was calculated, starting from the inoculation date (12 April 2012), by summing the daily mean air temperature using a 0°C base temperature.

Inoculation procedure

For each fungal isolate, subcultures were prepared on Petri dishes filled with PDA from the aforementioned stock cultures kept at -80°C. Conidial suspensions were obtained on the day before inoculation by flooding the surface of 5 day-old subcultures with distilled water and scraping the agar surface with a sterilized glass rod to release conidia (Suffert *et al.*, 2013). The suspensions were adjusted to a final concentration of 2.10⁵ conidia.mL⁻¹ using a Malassez counting chamber (Figure 1e). Two drops of surfactant (Tween20®, Sigma, France) were added to the suspensions to ensure adequate coverage of the inoculated leaf surface.

Conidial suspensions were applied along a 25 mm long section of the adaxial face of the two upper leaves (F1 and F2) of the main tiller of each plant. A paintbrush was used to apply each suspension, using a square plastic frame (25 × 25 mm) to delimit the surface to be inoculated. Then, the inoculated side of the leaf was turned over to avoid inoculum suspension trickling, and enclosed for 72 h in a transparent bag slightly wetted to maintain a high humidity and promote infection (Suffert *et al.*, 2013). The trial was set up as a randomized block design (216 plants) in a single greenhouse compartment. Each isolate × cultivar × leaf layer combination was replicated three times.

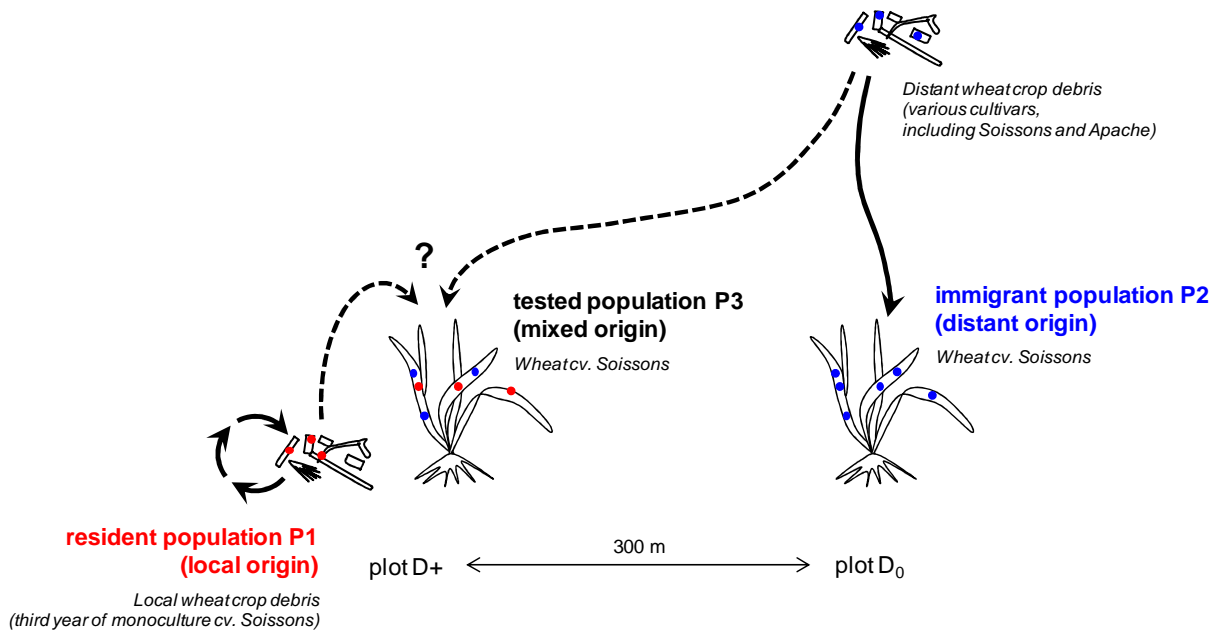


Figure 2 - Sketch of the origin of local *Zymoseptoria tritici* populations collected from wheat debris (P1) and from leaf lesion (P2, P3) during the early stages of a septoria tritici blotch epidemic in autumn 2009. The resident population (P1), considered as a sample of the local inoculum pool, was collected in a cv. Soissons wheat plot (D+; 3-year monoculture plot) from wheat crop debris. The immigrant population (P2), considered as a sample of an inoculum pool of distant origin, was collected in a cv. Soissons wheat plot without contaminated debris (D₀; previous crop oilseed rape) from lesions caused by inoculum coming from distant debris. The population P3 was postulated to have a mixed origin, that is, built up by ascospores ejected from debris of either local or distant origin, in an unknown proportion.

Assessment of maximum sporulating area and latent period

Disease progress, as well as maximum sporulating area and latent period (two components relevant for the characterization of aggressiveness in *Z. tritici*) were assessed as described elsewhere (Suffert *et al.*, 2013). The lesion size, estimated by the sporulating area (SPO), was visually assessed on each leaf (Figure 1f) with a hand lens (magnification $\times 10$) as the percentage of the inoculated leaf surface (1, 2, 3 and 5%, then 5% increments up to 100%) bearing pycnidia (Suffert *et al.*, 2013). The assessments were performed twice a week, by the same assessor, from the time of inoculation until leaf senescence (14 assessments). A Gompertz growth curve (Berger, 1981) was fitted to the values of SPO(t) recorded on each leaf (Suffert *et al.*, 2013):

$$SPO(t) = SPO_{\max} \exp(-B_{SPO} e^{-r_{SPO} t}) \quad (\text{eq. 1})$$

where SPO_{\max} is the maximum sporulating area, r_{SPO} is a rate parameter, and B_{SPO} is a position parameter. $LatSPO_5$, the time elapsed from inoculation to 5% of the maximum percentage of area bearing pycnidia (SPO_{\max}) was calculated using eq. 1 and expressed in degree-days (dd). Hereafter, for the sake of simplicity, SPO_{\max} and $LatSPO_5$, will be termed SP (sporulating area) and LP (latent period), respectively.

Data analysis

Firstly, the effects of cultivar (Soissons, Apache), leaf layer (F1, F2), pathogen population (P1, P2, and P3) and isolate (I01 to I36) were investigated in a factorial analysis of variance (Table 1). Isolate identity was nested as a random factor within pathogen population. The variance of the sporulating area (SP) and of the latent period (LP), assessed on three replicates per isolate and 12 isolates per population, was partitioned into sources attributable to isolate I(P), population P and cultivar C, taking into account the effect of the leaf layer L, according to the model:

$$Y_{ripl} = M + I(P) + P + C + L + I(P) \times C + I(P) \times L + P \times C + P \times L + C \times L + \epsilon_{ripl} \quad (\text{eq. 2})$$

where Y_{ripl} is the value of the sporulating area or of the latent period of isolate i , in population p , for cultivar c , on leaf layer l , for replicate r , and ϵ_{ripl} the error term

Secondly, the mean of the sporulating area and of the latent period was compared between populations (P1, P2 and P3), and then between cultivars (Apache and Soissons), with isolate I(P) and leaf layer L as factors (Student-Newman-Keuls test).

Thirdly, the mean of the sporulating area and of the latent period was compared between populations for each wheat cultivar (Student-Newman-Keuls test); similarly, the mean of the sporulating area and of the latent period was compared between cultivars for each population.

Curve fitting and subsequent data analysis were performed with the S-PLUS 6.3 software (Lucent Technologies, Inc., USA).

Results

The aggressiveness of the *Z. tritici* isolates strongly depended on the population from which they originated and on the cultivar on which they were inoculated (Table 1). The population effect was significant for the sporulating area ($P = 0.028$) while it was not for the latent period. Inversely, the cultivar effect was significant for the latent period ($P < 0.001$) while it was not for the sporulating area. The isolate and leaf layer effects were significant both for the sporulating area and the latent period ($P < 0.001$). Interactions between factors were not significant except for cultivar \times leaf layer ($P < 0.001$) for the sporulating area and for isolate \times cultivar ($P < 0.001$) for the latent period.

The sporulating area was significantly different between the three pathogen populations ($P < 0.05$; Table 2), while no significant difference was detected for the latent period. For each of the two cultivars tested, there was no difference between the three populations (Student-Newman-Keuls tests; Figure 3) either for the sporulating area ($P = 0.068$ for Apache and $P = 0.051$ for Soissons) or the latent period ($P = 0.145$ for Apache and $P = 0.482$ for Soissons). The latent period was significantly different between the two cultivars ($P < 0.01$; Table 2), while no difference was detected for the sporulating area.

The latent period was significantly different on cv. Apache and Soissons for P1 and P3 ($P < 0.001$), but not for P2 (Figure 3a). The latent period of isolates belonging to P1 and to P3 was longer when inoculated on Apache (476.7 dd and 492.1 dd, respectively) than on

Table 1 - Effect of population origin (P1, P2, P3), cultivar (Apache, Soissons) and leaf layer (F1, F2) on sporulating area (SP) and latent period (LP) of 36 isolates of *Zymoseptoria tritici*.

SP

Factor		df	Sum of squares	Mean square	F	P
Population ^a	P	2	3744.2	1872.1	3.99	0.028
Cultivar	C	1	180.4	180.4	1.02	0.314
Leaf layer	L	1	11229.4	11229.4	63.29	< 0.001
Isolate(Population)	I(P)	33	15452.6	468.3	2.64	< 0.001
Residual	R	219	38857.3	177.4		

LP

Factor		df	Sum of squares	Mean square	F	P
Population ^a	P	2	47867.5	23933.7	1.76	0.188
Cultivar	C	1	33728.1	33728.1	18.82	< 0.001
Leaf layer	L	1	49680.3	49680.3	27.73	< 0.001
Isolate(Population)	I(P)	33	449154.4	13610.7	7.60	< 0.001
Residual	R	217	388811.9	1791.7		

^a the "Population" effect was tested against "Isolate(Population)", with the F-ratio $F_p = \text{Mean Square}_P / \text{Mean Square}_{I(P)}$, such as the interactions $P \times C$ and $P \times L$.

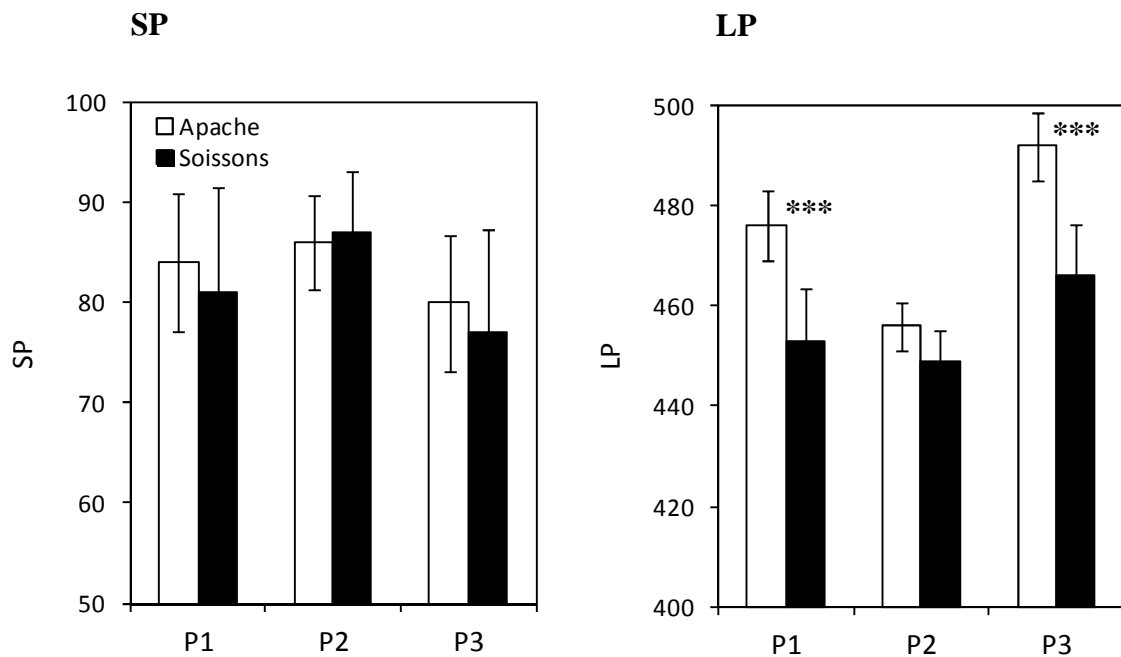


Figure 3 - Mean sporulating area (SP, % of the inoculated leaf area) and latent period (LP, days post inoculation) assessed for three *Zymoseptoria tritici* populations (P1, P2, P3) on two wheat cultivars (Apache and Soissons). *** means significant differences ($P < 0.001$) between the two cultivars for a given pathogen population (Student-Newman-Keuls tests); no difference was detected between the three populations for a given cultivar.

Table 2 - Mean sporulating area (SP) and latent period (LP) of *Zymoseptoria tritici* isolates belonging to three populations (P1, P2, P3), assessed on two wheat cultivars (Apache and Soissons).

Category	Variable	Population			Cultivar		
		P1	P2	P3	Apache	Soissons	
Sporulating area	SP ^a	82.8	86.6	79.1	83.5	82.3	ns
Latent period	LP ^b	465.3	453.1	480.0	474.8	456.3	***

^a expressed in % of the inoculated leaf area.

^b expressed in days post inoculation.

* result of Student-Newman-Keuls tests: ** : P < 0.05; *** : P < 0.001; ns : not significant.

Soissons (453.5 dd and 466.6 dd, respectively). Conversely, the sporulating area on cv. Apache and Soissons did not significantly differ between populations (Figure 3b).

Discussion

Evidence for local host adaptation of *Z. tritici* populations was provided in our experiment by significant differences in latent period detected between two pathogen populations: the immigrant population (P2) appeared to be similarly adapted to the sympatric (Soissons) and to the allopatric host (Apache), whereas the resident population (P1) appeared to be better adapted to the sympatric host. No difference in the two components of aggressiveness tested (latent period and sporulating area) was detected in the immigrant population between the two cultivars (Fig. 3): this population, despite collected on the sympatric host, was not particularly maladapted to the allopatric host. Filtration of the available distant inoculum by the sympatric host should be considered as negligible at the scale of a single infection cycle, because the two cultivars have been grown locally on wide areas for several years. The similar aggressiveness of the immigrant population assessed on the two cultivars also reflected the similar disease resistance rating of those two cultivars.

A resident pathogen population is usually adapted, at least to some degree, to its local host (Burdon 1993). When parasites migrate more than hosts within a metapopulation, local adaptation of parasites is expected, with a higher parasite relative fitness in sympatry than in allopatry (Kaltz & Shykoff, 1998; Laine, 2007). Several field experiments have established that populations of fungal plant pathogens are more aggressive on the cultivar from which they were isolated than on others (Clifford & Clothier, 1974; Kolmer & Leonard, 1986; Andrivon *et al.*, 2007; Pariaud *et al.*, 2009b). Our results are consistent with these findings, and, more specifically, with previous results obtained with *Z. tritici*. For example, *Z. tritici* isolates from California were found to induce more disease on Californian than on Oregonian wheat cultivars, while the reverse result was obtained for Oregonian isolates (Ahmed *et al.*, 1995). *Z. tritici* isolates were further showed to be better adapted to the host cultivar from which they originated than to other cultivars (Ahmed *et al.*, 1996). In our study, the absence of significant difference in mean latent period between the immigrant and the resident populations assessed on the sympatric host does not mean, however, that the resident population is better adapted to the sympatric host; rather, the susceptibility of the allopatric host appears to be lower to the resident population than the susceptibility of the sympatric

host. In other words, the resident population became less adapted to the allopatric host. Obtained at a very small spatio-temporal scale, this result could be explained by the joint growing of the two wheat cultivars in the same production area.

We established that a resident pathogen population locally evolving on a given wheat cultivar experienced, within few years, under repeated epidemic pressure, a fitness penalty on another host cultivar grown in the same area. Our result is consistent with those obtained by Zhan & McDonald (2004) which showed in a field experiment that immigrant isolates displayed nearly 10% lower fitness than the residents. Lower competitive ability of immigrants can arise from a trade-off in performance of isolates carrying alleles that are selectively advantageous in a population but deleterious in another because of environmental heterogeneity (Zhan & McDonald, 2013b). The 12 isolates belonging to the resident population collected during season 2009-2010 in the wheat monoculture plot resulted from a sexual cycle on the sympatric host, while their parents might have been present in that plot for one or two previous years and must have performed at least one asexual cycle during season 2008-2009. Therefore, the resident population had time to adapt to the sympatric host before it was sampled. Inversely, most of the 12 isolates belonging to the immigrant population, resulting from ascospores of a distant origin, did not have enough time to adapt much to the sympatric host before they were collected; in all likelihood, these 12 isolates originated from other wheat cultivars (the sympatric and allopatric hosts included, though) and experienced different evolutionary histories.

Consistent with the hypothesis of a more rapid pathogen evolution under monoculture (Zhan & MacDonald, 2013a), our findings emphasized the competitive disadvantage experienced by immigrants compared with residents, because they have not been selected for adaptation to the local environment (Giraud, 2006; Gladieux *et al.*, 2011). Most originally, we detected adaptation to the local environment at a smallest spatiotemporal scale than usually expected (Zhan *et al.*, 2002). Selection for quantitative traits in fungal pathogen populations has previously been shown to operate at the scale of a single-season epidemic and to result in a higher aggressiveness at the end of the crop season on the host genotype on which the pathogen population multiplied (Villaréal & Lannou, 2000; Cowger & Mundt, 2002). Here selection for quantitative traits operated within two consecutive years and led to the local, joint presence of pathogen populations adapted to the different host genotypes on which they multiplied: such populations can likely thrive a few hundred meters apart and be separated in time only by a single interepidemic period.

Although they were collected from the same wheat cultivar in the same agricultural basin, the isolates used in this study were genetically unique (unpublished data), as evidenced by SSR neutral markers genotyping developed by Gautier *et al.* (2014) and as expected from the well-known very high diversity of *Z. tritici* populations at a local scale (Linde *et al.*, 2002). The tested population (P3) consisted in isolates having a common history with the resident and the immigrant population, in a proportion to be determined: some isolates were "residents" while some others were "immigrants". The similarity in the aggressiveness profile in the tested and resident populations (Fig. 3) revealed that the evolutionary history of the tested population was closer to that of the resident than that of the immigrant population. Accordingly, the origin of inoculum for the tested population was more local than distant (Fig. 1): the main part of isolates belonging to the tested population would have been resident, resulting from the local inoculum pool, while a minor part would be immigrant. The early lesions that appeared in autumn 2009 in the monoculture plot were mostly caused by local primary inoculum, here ascospores produced on debris within the plot. This result is consistent with those obtained by Zhan *et al.* (1998), who estimated that 90 % of the isolates collected at the end of a field epidemic have a local origin (66% as asexual progeny and 24% sexual recombinants) and the remaining 10% are immigrants of distant origin. The results of our study are even more important than population genetic analyses based on neutral SSR markers usually did not allow to reveal a strong differentiation among populations collected at large or small spatial scales, whatever their history (Zhan *et al.*, 2001; Linde *et al.*, 2002).

Quantification of inoculum sources were previously investigated by assessing ascospores production directly from wheat debris in field (Suffert & Satche, 2011). This approach estimated the amount of locally produced inoculum able to infect more or less distant wheat fields. The reverse approach consisting in assessing the amount of airborne inoculum actually available for a target wheat field, for example by airborne trapping of ascospores (Bathgate & Loughman, 2001; Fraaije *et al.*, 2005; Duvivier *et al.*, 2013), cannot track the origin of inoculum. Mark-release-recapture, consisting in co-inoculating a wheat plot with selected *Z. tritici* genotypes, then separated from non-inoculant genotypes by using molecular genetic markers (Zhan & McDonald, 2013a), is a promising experimental approach. To test the inference presented in this study, wheat debris, produced by crossing under semi-controlled conditions different couples of parent isolates bearing rare alleles, could be placed at different distances from a target wheat crop. Debris would act as different, identified sources of inoculum. Such an approach would maximize the probability to identify

thereafter progeny isolates and so to quantify more accurately the different origins of inoculum.

A previous study (Suffert & Satche, 2011) showed that debris management options had a strong but transient effect on the dynamic of the subsequent *Septoria tritici* blotch epidemic: the more debris present on the soil surface, the more severe initial disease was, but differences vanished in early spring. While a "quantitative" management of the primary inoculum would probably not be efficient in Western Europe conditions, a "qualitative" management should be taken into consideration. Differential adaptation of local pathogen populations to allopatric vs. sympatric host cultivar, as highlighted in the present study, could be implemented in strategies based on host diversification at a local scale.

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Table S1 - Populations of *Zymoseptoria tritici* collected from wheat debris (P1, resident population) and from leaf lesions (P2, immigrant population; P3, tested population) in autumn 2009 during the early stages of a *Septoria tritici* blotch epidemic.

P1 ^a			P2 ^b			P3 ^b			
isolate code	date of collection	isolate code	date of collection	isolate code	date of collection	isolate code	date of collection	isolate code	date of collection
INRA09-FS0460	23 October 2009	INRA09-FS0729	24 November 2009	INRA09-FS0704	24 November 2009	INRA09-FS0704	24 November 2009		
INRA09-FS0501		INRA09-FS0731	30 November 2009	INRA09-FS0705		INRA09-FS0705			
INRA09-FS0502		INRA09-FS0732		INRA09-FS0706		INRA09-FS0706			
INRA09-FS0503		INRA09-FS0798	8 December 2009	INRA09-FS0707		INRA09-FS0707			
INRA09-FS0504		INRA09-FS0799		INRA09-FS0709		INRA09-FS0709			
INRA09-FS0506		INRA09-FS0800		INRA09-FS0710		INRA09-FS0710			
INRA09-FS0509		INRA09-FS0803		INRA09-FS0711		INRA09-FS0711			
INRA09-FS0510		INRA09-FS0805		INRA09-FS0712		INRA09-FS0712			
INRA09-FS0511		INRA09-FS0806		INRA09-FS0714		INRA09-FS0714			
INRA09-FS0512		INRA09-FS0809		INRA09-FS0715		INRA09-FS0715			
INRA09-FS0513		INRA09-FS0811		INRA09-FS0718		INRA09-FS0718			
INRA09-FS0514		INRA09-FS0813		INRA09-FS0722		INRA09-FS0722			

^a collected from ascospores ejected from perithecia produced on debris.

^b collected from pycnidiospores liberated from pycnidia produced on leaf lesions of wheat cv. Soissons

Conclusion générale et perspectives de la thèse

Conclusion générale et perspectives de la thèse

1. Rappel des objectifs et de la stratégie

L'objectif de la thèse était d'identifier les déterminants des phases épidémiques précoces de la septoriose du blé que sont la quantité, l'efficacité et l'origine de l'inoculum primaire. L'étude de chacun de ces déterminants a fait l'objet d'un chapitre. Une stratégie en quatre points a été adoptée. Tout d'abord, des questions pertinentes reliant chaque déterminant ont été posées. Ensuite, afin de répondre à ces différentes questions, plusieurs approches méthodologiques ont été combinées (épidémiologie quantitative, biologie moléculaire, génétique des populations et expression des interactions hôte-pathogène). Afin de travailler à des échelles de temps et d'espace en accord avec l'objectif général, permettant notamment de relier périodes épidémique et inter-épidémique, un dispositif au champ pluriannuel unique a été utilisé. Ce dispositif pluriannuel était constitué d'une monoculture de blé hébergeant une population résidante de *Z. tritici* suspectée d'être mieux adaptée aux conditions locales, en particulier à la variété de blé hôte (sympatrique), et une autre parcelle de blé hébergeant une population pathogène immigrante d'origine distante, au moins pour ce qui concerne les phases épidémiques précoces. Enfin, les résultats obtenus pour chaque déterminant ont été analysés indépendamment les uns des autres, avec des conclusions distinctes. Cette stratégie s'est avérée efficace. Dans la discussion générale qui suit, j'ai récapitulé les principaux résultats à retenir de chaque chapitre et je les ai considérés indépendamment les uns des autres. J'ai ensuite rapproché entre eux certains de ces résultats et retenu ceux qui, comme un faisceau de preuves cohérentes, m'ont permis de caractériser la façon dont commence une épidémie de septoriose. Le commencement et la fin des épidémies étudiées, ainsi que leur intensité, ont été replacés dans un cadre temporel plus large (sept années successives de maladie sur le dispositif pluriannuel de Grignon). Cet élargissement de perspective a été l'occasion de m'interroger sur les échelles de temps (annuelle vs. pluriannuelle) réellement pertinentes pour analyser ce qu'il est d'usage de considérer comme le commencement et la fin d'une épidémie.

2. Principaux résultats

Dans le second chapitre, l'inoculum primaire de *Z. tritici* présent dans l'air (ascospores) a pu être quantifié au cours de deux épidémies successives à l'intensité contrastée. Des différences

significatives de quantités d'ADN, converties en nombre d'ascospores collectées, ont été mises en évidence entre ces deux années, entre périodes épidémiques ("début du commencement" et "fin du commencement"), ainsi qu'entre parcelles en fonction de la présence locale ou non de résidus contaminés. Aucun lien manifeste n'a été établi entre la précocité des deux épidémies et la quantité d'inoculum primaire dans l'air ou la présence de résidus contaminés. Ce résultat, surprenant à première vue, est cohérent avec ceux obtenus précédemment qui ont montré que la quantité d'inoculum est un facteur peu limitant : il est toujours présent en quantité suffisante pour initier une épidémie, même si les attaques précoces sur les premières feuilles de blé peuvent être transitoirement un peu plus intenses en présence de résidus contaminés (Suffert & Sache, 2011). D'autres facteurs agissent sur le commencement des épidémies, tels que des conditions météorologiques plus ou moins favorables (principalement pluviométrie, humidité et températures modérées ; Arseniuk et al., 1998 ; Chilvers et al., 2007 ; Landschoot et al., 2011 ; Dawidziuk et al., 2012), ainsi que les niveaux d'attaque de l'année précédente qui conditionnent l'intensité de la reproduction sexuée et donc la quantité d'ascospores disponibles (Suffert et al., 2011 ; Duvivier et al., 2013). Les résultats des piégeages d'ascospores ont illustré ces effets temporels interannuels (Figure 1) : la première année étudiée (2011-2012) a succédé à une épidémie particulièrement peu intense (2010-2011), à l'inverse de la seconde (2012-2013). Des effets intra-annuels ont aussi été mis en évidence : la quantité d'ascospores présente au début des épidémies a varié avec la saison épidémique (accroissement jusqu'au milieu de l'hiver 2012-2013, puis diminution jusqu'au printemps) et le niveau de contamination des résidus (estimé par des tests d'éjection d'ascospores ; Suffert & Sache, 2011). J'ai pu bénéficier pendant ma thèse de deux années contrastées du point de vue de la pression d'inoculum et de l'intensité de l'épidémie, ce qui m'a permis de valider dans des conditions hétérogènes, mais bien réelles, la méthode de quantification des ascospores. Enfin, les résultats du chapitre II ont confirmé que des ascospores de *Z. tritici* sont présentes dans l'air de façon continue en automne et en hiver (Hunter et al., 1999 ; Bathgate & Loughman, 2001 ; Duvivier et al., 2013).

Les limites de détection et de quantification de la méthode moléculaire ont été établies et analysées dans le Chapitre II. Celles-ci doivent être discutées au regard de l'ensemble des études du même type ; selon certaines il serait possible de détecter, voire de quantifier, une ou deux spores (Carisse et al., 2009 ; Rogers et al., 2009 ; Parker et al., 2014), ce qui reste difficile à concevoir compte tenu des difficultés et limites méthodologiques rencontrées pendant ma thèse. L'extraction de l'ADN des ascospores comporte plusieurs étapes au cours

desquelles une fraction non négligeable des quantités d'ADN peut être involontairement éliminée, d'où l'importance du bon rendement d'extraction et de ne pas faire l'économie de témoins d'extraction (positifs et négatifs) validant la quantification. Quantifier implique détecter, mais l'inverse n'est pas forcément vrai. Il ressort des résultats de la thèse, compte tenu du contexte épidémiologique, que détecter la présence d'ascospores (en petites quantités) importe davantage que de les quantifier. L'objectif le plus pragmatique est certainement de se contenter d'un seuil de détection épidémiologiquement acceptable (suffisamment bas pour l'usage qui sera fait de l'information recueillie) et méthodologiquement robuste (10 à 20 ascospores), plutôt que de chercher à abaisser absolument le seuil de quantification (100 à 150 ascospores dans le cas présent) vers "une spore quantifiée". Pour cette raison, l'approche consistant à travailler sur les effectifs d'échantillons affectés à différentes classes (prenant en compte le Ct des trois répétitions), me semble plus pertinente que l'analyse des quantités d'ADN de la totalité des échantillons, sans que celles dont la fiabilité est discutable ne soient écartées.

Une autre limite de la méthode de quantification par qPCR concerne la viabilité des ascospores piégées (mortes ou vivantes et donc infectieuses), qui demeure inconnue. Une alternative consisterait à quantifier l'ARN des cellules (produit uniquement si les cellules sont viables) ; il s'agit toutefois d'une molécule instable et difficile à manipuler (West et al., 2008).

Le choix a été fait d'étalonner la méthode de quantification en utilisant des pycnidiospores de *Z. tritici* collectées *in planta* et facilement manipulables au laboratoire, plutôt que d'utiliser de l'ADN plasmidique (Duvivier et al., 2013). Cela a permis de raisonner en nombre de spores et de ne pas se limiter à des quantités d'ADN, ce qui m'a semblé pertinent dans la perspective de validation externe des résultats (comparaison de l'ordre de grandeur des quantités d'ascospores piégées avec celui des études précédentes ; Hunter et al., 1999 ; Bathgate & Loughman, 2001 ; Duvivier et al., 2013). La méthode de conversion utilisée est proche de celle de Duvivier et al. 2013. La conversion des quantités d'ADN de pycnidiospore en quantités d'ADN d'ascospore en se basant un rapport 1:3 (deux noyaux dans les ascospores contre quatre à huit noyaux dans les pycnidiospores ; Shaner, 2010) relève du bon sens, mais n'a pu toutefois être formellement validée. Il s'agit d'une limite importante de la stratégie que j'ai adoptée. Idéalement, des ascospores éjectées à partir de résidus contaminés auraient pu être utilisées, mais le caractère non répétable de l'opération et les difficultés méthodologiques que pose la quantification visuelle d'ascospores dans une solution

aqueuse ou alcoolique (nécessaire à la constitution d'une gamme) ont été trop contraignantes. La mise au point en 2013 d'une méthode de croisement de deux isolats parentaux de *Z. tritici* en conditions semi-contrôlées (F. Suffert, com. pers.), validée en 2014, est intervenue trop tard pour pouvoir être utilisée dans le cadre de la thèse.

Dans le troisième chapitre, les différences de pathogénicité entre les deux types de spores de *Z. tritici* (qui peuvent toutes les deux jouer le rôle d'inoculum primaire en cas de présence de résidus contaminés) ont été caractérisées. La production d'inoculum primaire à partir de résidus contaminés étudiés en tant que "source" avait déjà été estimée et comparée (ascospores vs. pycnidiospores ; Suffert & Sache, 2011). Dans le cas présent, la pathogénicité d'une pycnidiospore est ressortie comme étant supérieure à celle d'une ascospore. Cette différence correspond à une période de latence significativement plus longue après infection par une ascospore que par une pycnidiospore (environ 60 degrés-jour de différence, soit trois ou quatre journées au début de l'automne). La stratégie consistant à inoculer des feuilles de blé avec une suspension d'ascospores, puis avec des pycnidiospores collectées à partir de lésions issues de la contamination par les ascospores, a permis de s'affranchir de la variabilité entre individus parfois élevée pour certains traits d'agressivité (Suffert et al., 2013). D'autres champignons ascomycètes parasites de grandes cultures, comme *Pyrenopeziza brassicae* et *Leptosphaeria maculans* agents pathogènes du colza, ou *Didymella rabiei* agent pathogène du pois chiche, présentent des différences de pathogénicité entre les spores sexuées et asexuées (Karolewski et al., 2002 ; Li et al., 2004 ; Trapero-Casas et al., 2007). Une hypothèse a pu être formulée en comparant *Z. tritici* et ces autres champignons parasites. La différence de période de latence entre les pycnidiospores et les ascospores de *Z. tritici* pourrait être due à leur différence de taille, les spores les plus grosses étant suspectées d'avoir une capacité d'infection supérieure. La différence de période de latence entre les ascospores et les pycnidiospores pourrait avoir des conséquences importantes au cours des périodes épidémiques impliquant potentiellement les deux types de spores : au commencement de l'épidémie (si l'inoculum primaire est constitué à la fois d'ascospores et de pycnidiospores ; Suffert et al., 2011) ou pendant les deux derniers mois de l'épidémie (mai-juin) lorsque les étages foliaires supérieurs peuvent être infectés simultanément par des pycnidiospores (par splashing ; Shaw, 1987) ou par des ascospores produites sur des blés de l'année et dispersées par voie aérienne (Duvivier et al., 2013).

Dans le quatrième chapitre, divisé en deux parties, c'est l'origine de l'inoculum primaire qui a été étudiée. Deux stratégies ont été adoptées pour tenter de la déterminer. La première a consisté à analyser des changements dans la structure génétique de différentes sous-populations en utilisant des marqueurs neutres (SSR) récemment mis au point dans l'unité BIOGER (Gautier et al., 2014), la seconde en comparant leur profil d'agressivité *in planta*.

Aucune structure génétique n'a pu être identifiée au sein des différentes sous-populations de *Z. tritici* collectées pendant trois années successives, et ce malgré des populations légèrement plus différenciées en 2011-2012 qu'au cours des deux années précédentes. Ce résultat n'est pas vraiment surprenant compte tenu des connaissances acquises au cours des 20 dernières années sur le pathosystème blé-*Z. tritici*. J'ai notamment pu vérifier ce qui était jusqu'alors bien établi, à savoir une très grande diversité génotypique (Zhan et al., 2003 ; El Chartouni et al., 2011), un rapport des types sexuels MAT1-1 et MAT1-2 équilibré (Zhan et al., 2002 ; Boukef et al., 2012 ; Alloui et al., 2014), et une reproduction sexuée très active (Zhan et al., 1998 ; Cowger et al., 2008) y compris à une échelle spatiotemporelle fine (Chen et al., 1994 ; Linde et al., 2002). Une légère différenciation, telle que celle mise en évidence en 2011-2012, avait été établie dans quelques cas à des échelles de temps et/ou d'espace plus larges (Gurung et al., 2011 ; Drabesova et al., 2012), mais rarement à une échelle spatiotemporelle fine (Linde et al., 2002). La différenciation de sous-populations a priori directement issues de plusieurs cycles de reproduction asexuée (période épidémique) a été un peu plus élevée que celles issues de la collecte d'ascospores. Ce résultat paraît logique, mais rien ne laissait supposer que l'utilisation de marqueurs neutres puisse le montrer. Le fait que la différenciation des sous-populations issues de la collecte de pycnidiospores en fin d'épidémie 2011-2012 soit ressortie comme étant plus élevée que celles des sous-populations des deux années précédentes est un résultat intéressant car cohérent avec le contexte épidémiologique : à la faveur de conditions climatiques printanières favorables aux cycles de reproduction asexuée, l'épidémie de 2011-2012 a été plus intense que les deux précédentes. Par ailleurs, mes résultats ont remis en cause pour l'année 2011-2012 l'hypothèse selon laquelle les ascospores contribuent fortement au développement de la maladie en fin d'épidémie (Duvivier et al., 2013).

Onze marqueurs microsatellites ont été utilisés pour le génotypage des 800 isolats collectés (Gautier et al., 2014). Près de 1/5 des valeurs étaient manquantes (problèmes de

contamination ou d'erreurs de géotypage dues à des ADN extraits de mauvaise qualité). Il est probable que l'absence de ces données n'a eu que peu d'influence sur les résultats de structure obtenus.

Au final, l'approche de génétique des populations que j'ai adoptée n'a pas permis de répondre à la question initialement posée dans le chapitre IV. Les résultats obtenus n'ont pas permis d'établir de différences entre les sous-populations résidentes (locales) et immigrantes (distantes), ni d'établir de discontinuité génétique entre sous-populations de fin (saison n) et de début d'épidémie ($n+1$), et donc de déterminer l'origine de l'inoculum.

En s'appuyant sur le même dispositif expérimental au champ, le profil d'agressivité d'une population, dont l'origine était à déterminer, a été estimé et comparé à celui d'une population résidente (ascospores issues de résidus présents localement) et celui d'une population immigrante (lésions provoquées par des ascospores d'origine distante). J'ai montré que le profil d'agressivité de la population testée était plus proche de celui de la population résidente que de celui de la population immigrante, et conclu que l'épidémie avait été déclenchée majoritairement par de l'inoculum primaire local (ascospores issues des résidus). La généralisation de ce résultat à l'ensemble des parcelles de blé en monoculture, et notamment celles faisant l'objet d'un semis direct, devra faire l'objet de travaux supplémentaires. J'ai par ailleurs illustré le phénomène d'adaptation locale d'un agent pathogène à son hôte, conséquence de la spécialisation d'une population pathogène aux variétés hôtes les plus fréquentes (Gandon & Van Zandt, 1998), et mis en évidence la maladaptation de la population résidente vis-à-vis d'un hôte allopatrique (Kaltz *et al.*, 1999).

3. Intérêt de combiner différentes approches méthodologiques

Différentes approches ont été développées, dont certaines pour répondre à une seule et même question. Dans le cadre de la problématique générale de la thèse (caractérisation de l'inoculum primaire de *Z. tritici* et son rôle dans les phases précoces de la septoriose), la complémentarité de certains résultats m'a permis de déterminer quels facteurs étaient susceptibles d'influencer le commencement d'une épidémie et selon quelles modalités. Globalement, j'ai pu constater que certaines quantités (comme le nombre d'ascospores présentes dans l'air ou directement produites à partir de résidus contaminés) étaient finalement assez peu informatives, et que la

caractérisation d'effets qualitatifs (comme l'adaptation des populations à leur hôte, liée à leur historique épidémiologique et leur trajectoire évolutive associée) pouvait être plus pertinente. C'est bien la complémentarité des différentes approches méthodologiques qui m'a permis de faire progresser les connaissances sur le rôle de l'inoculum primaire dans les phases précoces d'une épidémie de septoriose. En effet, ni l'approche quantitative (en déterminant le nombre d'ascospores dans l'air), ni les outils de génétique des populations (par des marqueurs neutres SSR) n'ont permis à eux seuls d'identifier l'origine de l'inoculum primaire. Dans le cas de populations aussi diversifiées que *Z. tritici*, soumises à une phase de reproduction sexuée très étalée dans le temps, il n'est pas surprenant que ce soit l'approche expérimentale basée sur la comparaison de profils d'agressivité entre populations résidante qui se soit révélée concluante (chapitre IVb).

Les effets de la présence locale de résidus contaminés ont été mis en évidence par l'analyse de l'adaptation différentielle de populations de *Z. tritici* à leur hôte (cultivars sympatrique et allopatrique) en fonction de leur origine (Chapitre IVb). Les effets de ces résidus n'ont en revanche pu être établis ni par l'approche qualitative de génétique des populations (Chapitre IVa) ni par l'approche d'épidémiologie quantitative (Chapitre II). La question du rôle des ascospores en tant qu'inoculum secondaire au printemps, comme suggéré par Duvivier et al. (2013), n'était pas au centre des préoccupations de la thèse. Plusieurs de nos résultats ont toutefois apporté des éléments de réponse. J'ai montré que des ascospores étaient présentes dans l'air au-dessus des parcelles de blé jusqu'en mars-avril, et ce y compris l'année 2011-2012 caractérisée par une épidémie de septoriose de faible intensité (Chapitre II). Combinées à ce résultat, les estimations du taux de différenciation des sous-populations collectées en 2011-2012 (Chapitre IVa) suggèrent que l'impact des ascospores en tant qu'inoculum secondaire est limité puisque les populations collectées au printemps et en fin d'épidémie cette même année étaient plus différenciées (valeurs de F_{ST} faibles et inférieures à 0,1, mais tout de même significativement supérieures) que les sous-populations des deux années précédentes issues de pycnidiospores ou d'ascospores. Cette différence a été interprétée comme étant la conséquence de cycles de reproduction asexuée plus nombreux et d'un rôle négligeable des ascospores au printemps 2012. Ces résultats ne sont pas incompatibles avec l'implication tardive d'ascospores en mai-juin, certaines années, dans la contamination des étages supérieurs du couvert.

L'analyse des deux expérimentations conduites sur plantes adultes en conditions contrôlées (Chapitre III et Chapitre IVb) a porté sur deux ou trois traits d'agressivité (capacité de sporulation, période de latence, taille des lésions), qu'une précédente étude avait établi comme étant les plus pertinents (Suffert et al., 2013). Dans les deux cas, des différences significatives entre modalités ont été obtenues pour la période de latence mais pas pour les autres traits d'agressivité. D'un point de vue méthodologique, ceci montre que la période de latence, bien que complexe à estimer (nécessité de suivre une cinétique), est une variable indispensable dans les études sur *Z. tritici*. D'un point de vue épidémiologique, la période de latence apparaît comme un trait d'agressivité variable et donc potentiellement soumis à sélection. Cette conclusion est cohérente avec les résultats d'une expérimentation récente qui suggèrent l'existence de processus de sélection favorisant, pendant la montée de la maladie dans le couvert, les individus ayant une période de latence plus courte (F. Suffert, com. pers.).

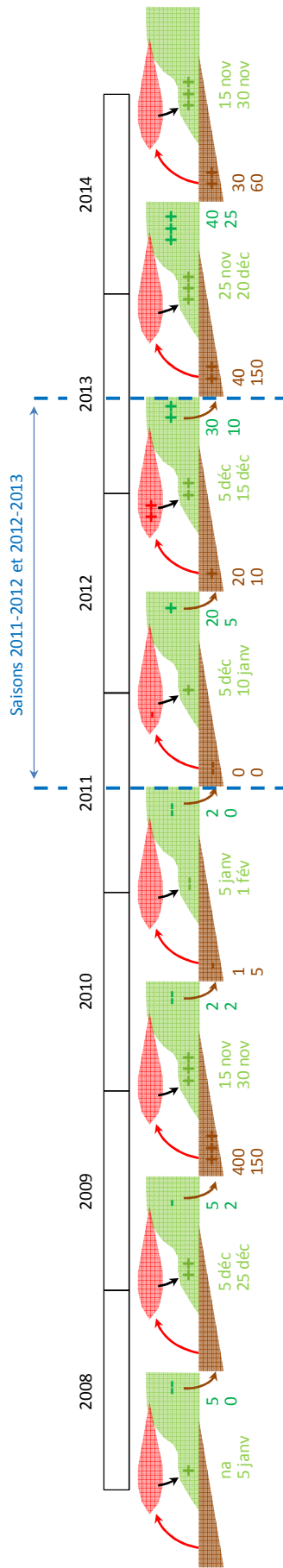
4. Le commencement d'une épidémie, de l'échelle annuelle à l'échelle pluriannuelle

Les résultats de ma thèse ont porté sur deux années d'épidémie, voire trois, selon les chapitres. Le dispositif utilisé, une parcelle de blé en monoculture depuis 2007 et une parcelle de blé distante de 400 m dont l'emplacement a changé chaque année, a fait l'objet d'un suivi épidémiologique régulier et continu. À ce stade de la réflexion, j'ai estimé pertinent de replacer mes résultats dans un cadre temporel plus large, à savoir la succession de plusieurs épidémies annuelles, pour comprendre comment une épidémie de septoriose (notamment son commencement) est influencée par l'épidémie précédente (notamment sa fin).

Les résultats quantitatifs obtenus au chapitre II ont permis d'estimer, pour chacune des deux années étudiées (parcelle en monoculture avec résidus ; 2011-2012 et 2012-2013), quatre indicateurs de pression pathogène : intensité de la reproduction sexuée sur les résidus, quantité d'ascospores présentes dans l'air au dessus de la parcelle, précocité de l'épidémie, sévérité d'attaque en fin d'épidémie (Figure 1). À cette occasion, les indicateurs ont été estimés pour les quatre épidémies précédentes (2007-2008, 2008-2009, 2009-2010, 2010-2011) et la suivante (2013-2014) en exploitant les données acquises par F. Suffert à partir du dispositif pluriannuel. Il ressort d'une analyse empirique globale que les épidémies de septoriose 2011-2012 et 2012-2013 ont été contrastées en ce qui concerne la quantité

d'inoculum et l'intensité de maladie observée, mais aussi qu'elles ont été représentatives de celles observées pendant les huit années considérées. L'exploitation des notations de maladie au champ (incidence et sévérité des attaques) pour sept années et du niveau de contamination des résidus (quantité d'ascospores éjectées) pour six années a pu compenser l'absence de quantification des ascospores présentes dans l'air en dehors des deux années étudiées pendant ma thèse. La quantité d'inoculum, quelle que soit la nature de l'indicateur de pression pathogène considéré (Figure 1), s'est progressivement accrue entre le début de l'année 2011, où la quantité d'ascospores retrouvée dans les résidus était nulle (--), et la fin de l'épidémie en 2013 caractérisée par une intensité d'attaque élevée des étages foliaires supérieurs (++). Ces résultats suggèrent qu'en l'absence de conditions climatiques très défavorables à la maladie, comme ce fut le cas pendant la période considérée (hiver et printemps relativement pluvieux), la pression pathogène ne décroît pas à court terme et le passage d'une phase épidémique à l'autre ne semble pas limité (reproduction sexuée et survie sur résidus → contaminations précoces à la levée → survie hivernale sur plante → montée au sein du couvert par splashing → intensification de la maladie à l'échelle d'un étage foliaire → contamination des étages supérieurs par des vols tardifs d'ascospores ; Figure 6, Chapitre I).

La représentation graphique de l'évolution des quatre indicateurs de pression pathogène, convertis en indice (-1 à +3), au cours des sept années fait état d'une remarquable continuité temporelle (Figure 2). L'indice de pression pathogène a en effet été très variable entre 2008 et 2010 avant de s'établir à un niveau très faible (-1) en 2010 et 2011 : attaque relativement tardive, épidémie peu sévère et faible intensité de reproduction sexuée (résidus probablement peu contaminés). Entre 2011 et 2015, période qui inclut les deux années étudiées pendant ma thèse, l'indice de pression pathogène a régulièrement augmenté pour atteindre son niveau maximal (+3) au cours des deux dernières épidémies. Une discontinuité temporelle, définie ici par la hausse ou la baisse d'au moins trois points de deux valeurs consécutives de l'indice, a été mise en évidence en 2009 (intensité de reproduction sexuée très élevée sur des résidus pourtant issus d'une épidémie dont l'intensité d'attaque finale était faible) et en 2009-2010 (épidémie très précoce précédée d'une intensité d'attaque très faible à la fin de l'épidémie précédente). La première discontinuité pourrait s'expliquer par des conditions automnales particulièrement favorables à la reproduction sexuée, mais cette hypothèse reste invérifiable. La seconde discontinuité s'explique, plus aisément, par des conditions climatiques printanières très défavorables à la septoriose, qui ont marqué un coup d'arrêt à son développement dans les parcelles entre mars et mai 2010.



Quantité d'ascospores présentes dans l'air au dessus de la parcelle (estimation semi-quantitative : -- très faible ; +++ très élevée)

Sévérité d'attaque en fin d'épidémie de l'année n (estimation semi-quantitative : +++ très forte ; -- très faible)

a = estimation de la sévérité d'attaque moyenne (% de surface foliaire attaquée) sur la feuille F2 au 10 juin

b = estimation de la sévérité d'attaque moyenne sur la feuille F1 (drapeau) au 10 juin

Précocité de l'épidémie de l'année n (estimation semi-quantitative : +++ très précoce ; - très tardive)

date1 = date à laquelle l'incidence de la septoriose atteint 5% (au moins une lésion sur 1/20 des premières feuilles L1)

date2 = date à laquelle l'incidence de la septoriose atteint 100% et la sévérité d'attaque moyenne sur la feuille L1 atteint 20%

Intensité de la reproduction sexuée sur les résidus de l'année n-1 (estimation semi-quantitative : très faible ; très élevée)

c = nombre d'ascospores par g de résidus collecté pendant la première quinzaine d'octobre

d = nombre d'ascospores par g de résidus collecté pendant la première quinzaine de décembre

Figure 1 - Représentation de la dynamique épidémique de la septoriose causée par *Zymoseptoria tritici* dans une parcelle en monoculture de blé (domaine expérimental de Thiverval-Grignon) au cours de sept saisons successives (2008 à 2014). Quatre indicateurs de pression pathogène ont été définis (intensité de la reproduction sexuée sur les résidus, quantité d'ascospores présentes dans l'air au-dessus de la parcelle, précocité de l'épidémie, sévérité d'attaque en fin d'épidémie) ; la plupart des quatre indicateurs a été estimée pour chacune des sept saisons à partir de prélèvements et mesures réalisés sur le terrain (Suffert et al., 2011; Suffert, données non publiées), avec un effort particulier pour les deux saisons ayant fait l'objet de la thèse (2011-2012 et 2012-2013) ; les estimations chiffrées (partie basse de la figure) ont été transformées en classes : --, -, +, ++ et +++ (voir légende).

Le début d'une épidémie est habituellement défini par le moment où les premiers symptômes apparaissent sur la culture de l'année (par exemple début décembre en ce qui concerne la septoriose du blé) et sa fin par le moment ultime où la maladie est encore présente sur la culture (en juillet, mois de la moisson). La discontinuité, ou rupture temporelle, peut être caractérisée à la fois de façon quantitative (forte diminution de la présence de l'agent pathogène) et qualitative (changement possible de la structure des populations et remplacement d'une sous-population par une autre). Contrairement aux parasites biotrophes, comme les agents pathogènes des trois rouilles du blé (*Puccinia triticina*, *P. striiformis* et *P. graminis* f. sp. *tritici*), les parasites nécrotrophes ou hémibiotrophes comme *Z. tritici* peuvent survivre en l'absence de leur hôte principal (ou, le cas échéant, alternant) pendant la période d'inter-culture. La mise en évidence d'une discontinuité qualitative chez la rouille brune du blé, caractérisée par un mode de reproduction clonal et l'existence d'un nombre limité de pathotypes, est actuellement étudiée dans l'équipe Epidémiologie dans le cadre du projet PlantFoodSec (I. Sache, com. pers.). En ce qui concerne *Z. tritici*, il m'a été impossible de distinguer différentes sous-populations et d'identifier des changements génétiques ; seule l'étude de la discontinuité quantitative a été concluante. Cette réflexion sur la discontinuité de pression pathogène conduit à se poser la question de la définition des limites temporelles d'une épidémie : coïncident-t-elles forcément avec celles de la saison culturale ? *Z. tritici* est présent et actif sur résidus de culture pendant la période inter-épidémique ; il y réalise même une partie importante de son cycle biologique, à savoir la reproduction sexuée. La période d'inter-culture ne marque donc pas la fin du développement de l'agent pathogène. En utilisant l'indicateur de pression pathogène, on pourrait donc considérer que, dans la parcelle en monoculture de blé, l'épidémie de septoriose a débuté à la fin de l'automne 2011 (épidémie modérément précoce) et qu'elle s'est poursuivie jusqu'à l'hiver 2014-2015 (pas de baisse de la pression pathogène à ce jour). Au final, deux conceptions du commencement et de la fin d'une épidémie pourraient coexister en fonction de l'échelle temporelle considérée et des caractéristiques biologiques de l'agent pathogène. Dans la première définition, le commencement serait conditionné par la présence de l'hôte (apparition des premiers symptômes visibles sur les feuilles) ; il s'agit de la vision utilisée tout au long de ma thèse pour analyser les données. Dans la seconde définition, le commencement serait défini par la discontinuité de pression pathogène, indépendamment de la présence de l'hôte. Ces considérations pourraient avoir des conséquences au-delà de la simple représentation des épidémies, mais aussi au niveau de leur gestion.

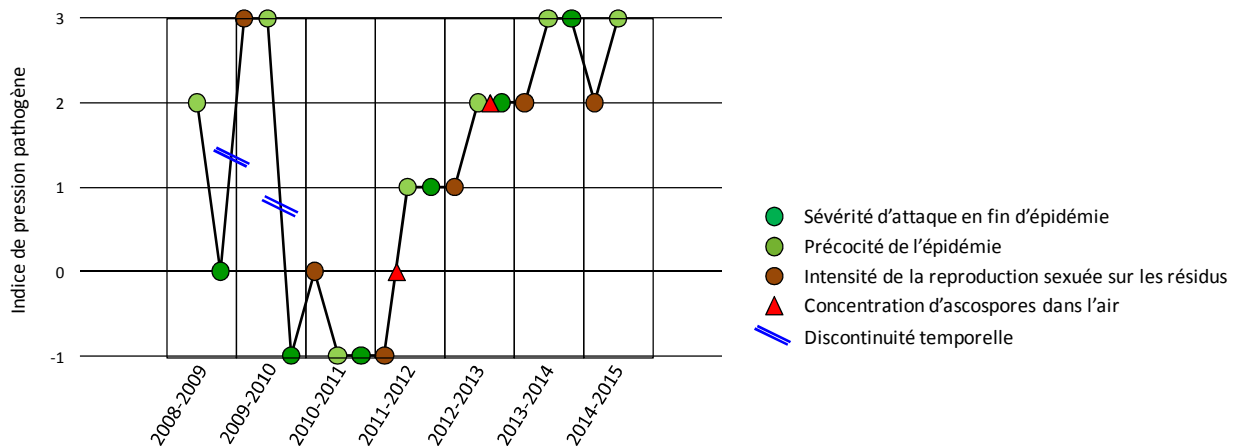


Figure 2 - Continuité temporelle de la pression pathogène de *Zymoseptoria tritici* dans une parcelle en monoculture de blé (domaine expérimental de Thiverval-Grignon) au cours de huit saisons successives (2008 à 2014) estimée à l'aide d'un indice quantitatif unique reprenant ceux définis Fig. 1 (intensité de la reproduction sexuée sur les résidus, quantité d'ascospores présentes dans l'air au-dessus de la parcelle, précocité de l'épidémie, sévérité d'attaque en fin d'épidémie) ; l'échelle semi-quantitative (-- à +++) a été convertie en une échelle numérique (-1 à 3) ; une discontinuité temporelle a été définie par la hausse ou la baisse d'au moins trois points de deux valeurs consécutives de l'indice.

5. Conséquences et applications pratiques d'une meilleure caractérisation de l'inoculum primaire

La principale application possible des résultats de ma thèse concerne la gestion des épidémies de septoriose. D'un point de vue quantitatif, les résultats que j'ai obtenus confirment que gérer (c'est-à-dire diminuer ou limiter) la quantité d'inoculum primaire par une réduction de la quantité de résidus est, à une échelle locale, illusoire : l'inoculum primaire peut avoir une origine distante et, de manière générale, sa quantité est rarement limitante, en début d'épidémie. Les différences de précocité entre parcelles avec résidus (semis direct en monoculture de blé) et sans résidus ne sont pas vraiment significatives ; il est probable que dans des rotations conventionnelles, l'influence de la présence de résidus contaminés sur une parcelle de blé soit insignifiante (Suffert & Sache, 2011). D'un point de vue qualitatif en revanche, certains effets mis en évidence dans la thèse pourraient être exploités, comme l'adaptation (et la maladaptation) de l'inoculum à l'hôte. L'alternance de variétés pourrait avoir un impact positif à une échelle locale (exploitation agricole, bassin de production) alors que ces effets ont généralement été mis en évidence à une échelle plus large (Papaïx et al., 2011; Papaïx et al., 2014). Pour résumer, une gestion quantitative de l'inoculum primaire serait probablement très peu efficace (à moins de supprimer toutes les sources d'inoculum sur de très grandes surfaces, perspective utopique), tandis que sa gestion qualitative mériterait d'être envisagée. L'adaptation différentielle de populations pathogènes à leurs hôtes pourrait être prise en compte dans des stratégies de protection basées sur l'alternance temporelle des variétés utilisées localement.

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Titre - Les déterminants des phases précoces des épidémies de septoriose du blé (*Zymoseptoria tritici*) : quantité, efficacité et origine de l'inoculum primaire

Résumé - Les maladies foliaires fongiques sont une contrainte majeure des systèmes céréaliers. Elles constituent par ailleurs de précieux modèles d'étude pour comprendre comment commence une épidémie, questionnement récurrent en épidémiologie végétale. Dans cette thèse ont été étudiés trois des principaux déterminants des phases précoces des épidémies de septoriose du blé causée par *Zymoseptoria tritici* : quantité, efficacité et origine de l'inoculum primaire. L'étude a porté sur deux épidémies successives dans deux parcelles de blé proches l'une de l'autre au sein d'un dispositif pluriannuel (2011-2013 ; Grignon, France). La première parcelle, caractérisée par l'absence de résidus, était exposée à des sources distantes d'inoculum primaire, et la seconde, en monoculture de blé depuis plusieurs années, présentait une quantité importante de résidus contaminés agissant comme source locale d'inoculum primaire. Dans la première partie de la thèse, la capture d'ascospores à l'aide d'un piège volumétrique couplé à une quantification de l'ADN par qPCR a permis de quantifier l'inoculum primaire présent dans l'air au-dessus des parcelles pendant les phases épidémiques précoces. Les limites de détection et de quantification ont été déterminées, discutées et utilisées afin d'estimer de faibles quantités d'ascospores. Ni la présence locale de résidus contaminés ni la quantité d'inoculum au-dessus des parcelles n'ont été corrélées avec la précocité des deux épidémies. Dans la seconde partie, la pathogénicité des ascospores et des pycnidiospores de *Z. tritici* a été estimée sur plante adulte et comparée. La période de latence consécutive à une infection par ascospore a été plus longue de 60 degrés-jour qu'après une infection par une pycnidiospore. Dans la troisième partie de la thèse, qui visait à identifier l'origine de l'inoculum primaire, deux stratégies ont été adoptées. La première stratégie a consisté à rechercher des changements dans la structure génétique de différentes sous-populations collectées dans les deux parcelles sur une période de trois années en utilisant des marqueurs neutres (SSR). Aucune structure n'a été identifiée, mais de légères différenciations chez certaines sous-populations, cohérentes avec le contexte épidémiologique (nature du cycle de reproduction dont elles découlent, période épidémique, intensité de maladie), ont été mises en évidence. Ces résultats n'ont toutefois pas permis de déterminer l'origine de l'inoculum primaire puisqu'aucune différence entre les sous-populations résidentes (locales) et immigrantes (distantes), ni de discontinuité génétique entre sous-populations de fin (saison n) et de début d'épidémie (saison n+1), n'a été mise en évidence. La seconde stratégie a consisté à comparer le profil d'agressivité (capacité de sporulation et période de latence) d'une population de *Z. tritici* collectée en début d'épidémie dans la parcelle en monoculture de blé, à celui d'une population résidente (ascospores issues des résidus présents dans la parcelle) et d'une population immigrante (lésions provoquées par des ascospores d'origine distante). Le profil de la population testée, plus proche de celui de la population résidente, a suggéré que l'épidémie avait été déclenchée majoritairement par un inoculum primaire d'origine locale. Il ressort de cette étude de cas qu'une gestion quantitative (réduction) de l'inoculum primaire serait probablement très peu efficace, tandis que sa gestion qualitative, tenant compte de l'adaptation différentielle de populations pathogènes à leurs hôtes, mériterait d'être intégrée dans des stratégies de protection (alternance de variétés dans le temps). Pour finir, la définition du début et de la fin d'une épidémie a été discutée, selon que l'on se place à une échelle annuelle (absence de l'hôte comme critère de début/fin) ou pluriannuelle (discontinuité de pression pathogène comme critère de début/fin, indépendamment de l'absence de l'hôte).

Mots clefs - ascospore, *Mycosphaerella graminicola*, profil d'agressivité, population immigrante, pycnidiospore, qPCR, quantification, résidus.

Title - Components of the early stages of septoria tritici blotch epidemics (*Zymoseptoria tritici*): quantity, efficiency and origin of primary inoculum

Abstract - Fungal foliar diseases are a major constraint to cereal production systems. They constitute relevant models to elucidate how an epidemic begins, which is a recurrent question in plant disease epidemiology. In this thesis three of the main components of the early stages of the epidemics of septoria tritici blotch, a wheat disease caused by *Zymoseptoria tritici*, were studied: the quantity, the efficiency and the origin of primary inoculum. The study focused on epidemics occurring over the 2011-2013 cultural seasons in two wheat plots located close to each other in Grignon (France). The first plot, characterized by the absence of debris, was exposed to distant sources of primary inoculum, while the second plot, grown in wheat for several years, had a large amount of contaminated debris acting as a local source of primary inoculum. In the first part of the thesis, ascospore trapping using a volumetric spore trap combined with DNA quantification by qPCR allowed to quantify the inoculum present in the air above the wheat plots during the early stages of the epidemics. The limits of detection and quantification were determined, discussed and used to estimate small amounts of ascospores. Neither the local presence of contaminated debris nor the quantity of inoculum above the plots was correlated with the earliness of the epidemics. In the second part, the pathogenicity of *Z. tritici* ascospores and pycnidiospores was estimated on adult plants. The latent period following infections by ascospores was 60 degree-days longer than following infection by pycnidiospores. In the third part of the thesis, which aimed to identify the source of primary inoculum, two strategies were developed. The first strategy investigated changes in the genetic structure of different subpopulations collected from both wheat plots over a period of three years using neutral markers (SSR). No structure was identified, but slight differentiations in some subpopulations, consistent with the epidemiological context (nature of the reproduction cycle from which they derived, epidemic period, disease intensity) were highlighted. These results, however, did not allow to determine the origin of the primary inoculum because no difference between resident (local) and immigrant (distant) subpopulations, or a genetic discontinuity between subpopulations from the end (season n) and the beginning of epidemics (season n + 1), were identified. The second strategy compared the aggressiveness profile (sporulation capacity and latent period) of a *Z. tritici* population collected at the beginning of an epidemic in the monoculture wheat plot and of a resident population (ascospores ejected from debris present in the plot) and to an immigrant population (leaf lesions caused by ascospores of distant origin). The profile of the tested population, closer to that of the resident population, suggested that the epidemic was mainly initiated by primary inoculum of local origin. This case study showed that a quantitative management (reduction) of the primary inoculum would be probably inefficient, while its qualitative management, taking into account the differential adaptation of pathogen populations to their hosts, deserves to be taken into account in crop protection strategies (alternating wheat cultivars in time). Finally, the definition of the beginning and the end of an epidemic was discussed, depending if we consider an annual scale (absence of the host as a beginning or end criterion) or a multiannual scale (pathogen pressure discontinuity as the beginning or end criterion, regardless of the absence of the host).

Keywords - ascospore, *Mycosphaerella graminicola*, aggressiveness profile, immigrant population, pycnidiospore, qPCR, quantification, wheat debris.