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Course handout:

Phytopathological Diagnostics

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Introduction

Diagnosis of plant disease is dependent mainly on observations of the syptoms of the disease and the presence of a pathogenic agent in or on diseased tissues. There are three symptom types that are characteristic of plant disease.

a. Necrisis or death of infected tissue-leaf spot.

b. Hyperplasia and hypertophy- increase in cell number and size respectively.

c. Hypoplasia and hypotrophy-reduced growth and stunting, characteristicof plant attached by parasitic nematodes. The major agents producing these symptom types are either fungi, bacteria, viruses, nematodes, insects or so-called physiological disorders or non parasitic disease agents. The determination of the causal agent of a disease is usually the first step in attempts to control the disease. It sometimes felt that diagnosis is simple matter of looking at the plant and immediatly the disease is known and complete recommendation can be given for control. Consequently a carful evaluation of the total situation is necessary before a correct diagnosis is made.

At present, diagnostic methods in the laboratory are based mainly on the direct examination of samples and the identification of colonies obtained by culture. Direct examination, although of low sensitivity, has the advantage of providing a quick response (within hours). The detection of fungal DNA by PCR (Polymerase Chain Reaction) is develops rapidly so as to be either specific to a given genus or species or panfungal. This last technique, coupled with sequencing, has been progressing in research laboratories since the Consortium for the Barcode of Life (CBOL) defined the gene encoding for the ITS region (Internal Transcribed Spacer) as a universal fungal marker. In addition, ELISA is an immuno-enzymatic method for the detection of pathogens based on the recognition by specific antibodies of the envelope proteins of viruses. It helps diagnose leafroll viruses or bacteria affecting plants. The identification of the causal agent requires the implementation of techniques that are less sensitive than for detection, but with levels of specificity such that they can be used to characterize this agent with greater precision, which is appropriate to the problem under consideration. Advances in molecular biology have led to the emergence of numerous techniques using the characterization of immunogenetic molecules (particularly proteins) or nucleic acid sequences. These methods can identify pathogens accurately, often before symptoms appear, facilitating early intervention.

This document dealt with several areas that are related to each other, through which it is possible to determine the pathogen by observing the symptoms that appear on the plant's organs. The identification of a phytopathogen including bacteria, fungi and virus is a crucial process in plant pathology to diagnose plant diseases and implement effective management strategies. This process can involve several steps and methods, ranging from conventional to innovative molecular methods.

This handout is divided into four chapters which are organized as follows :

Chapter I : Presents the different methods of detection of plant diseases, their principles, their advantages, by combining traditional diagnostic techniques with advanced molecular methods.

Chapter II : Describes the steps of isolation, purification, identification of phenotypic characteristics of bacteria, using conventional biochemical tests and by extraction of genomic DNA and identification by advanced molecular methods.

Chapter III : Is devoted to clearly presenting the methods of detection of viruses such as tests on indicator plants, electron microscopy and techniques of RT-PCR and sequencing.

Chapter IV : determines the identification keys of biological aggressors and highlights the different types of traps and sampling methods (insects and nematodes).

Chapter I : Fungal diagnosis

I. 1. Conventional methods (microscopic observation, cultures)

I.1.1. The generalist diagnosis

The generalist diagnosis initially allows us to characterize the symptoms (type of symptoms, description – distribution on the tree and in the plot) and to hypothesize on the origin of the disease.

The first step is to make a visual survey on the ground. Sometimes the diagnosis is made on the basis of visible symptoms or directly on the presence of organisms or signs of recognition (rot, mycelium, spores, vector...) (Grabowski, 2008). It should also be emphasized that the identification of the origin of a symptom requires experience and good knowledge in plant pathology : the plant species, the pathogens recognized for this species, the biology and life cycle of pathogens. In this case, the report is delicate and requires more information such as :

- the characteristics of the plot (sun exposure, wind, rain) ;

- soil structure and agronomic factors (treatment, amendment, watering

cultural background...) ;

- the spatial distribution and course of the disease (localized, random or uniform) ;

- detailed description of the symptoms (affected area, appearance, color, deformity, etc.).

In general, injuries or degradations of non-parasitic origin (hail, frost, toxicity) are generalized over the entire plot or follow a symmetrical axis (row, border). In the case of parasitic diseases, the distribution is rather scattered or concentrated, with progressive symptoms (Schubert et al., 1988).

I.1.1.1 Description of symptoms

Symptoms provide excellent clues to determine the cause of a disorder or at least to clarify whether the factor responsible is biotic or abiotic. At first glance, our attention is directed towards the part of the plant presenting the main symptom. If dieback is observed in plants.

A. Affected parts of plant

It is therefore essential to examine more closely all the parts of the plant : the stem, the vascular system, the crown and the roots. Is dieback related to a fungal or bacterial disease affecting the vascular system, roots or crown, damage by nematodes, winter frost, phytotoxicity by a pesticide, root asphyxia due to excess water or compact soil, to high soil salinity, over-fertilization, lack of water, damage by an insect...

B. Pattern of symptoms

The pattern of symptoms on affected parts of the plant is an excellent clue to the origin of the problem or at least to determine whether the factor involved is biotic or abiotic. Regular burns at the leaf margin or between the veins promote the involvement of a non-parasitic factor, such as excessive salinity in the soil, mineral deficiency, significant water loss through the foliage on a hot day, dry and windy, damage to the vascular system caused by winter frost hindering the water supply of the foliage (Figure 1), phytotoxicity due to a pesticide , ect.

Figure 1: (A) : Straight, uniform and regular burn at the margin of leaflets caused by high soil salinity; (B) :Burns at the apex of leaflets with an appearance similar from one leaflet to another. Problem related to poor translocation of calcium to the young leaves (Lacroix, 2004)

The same applies to the presence of stains of regular shape and size uniformly distributed on the foliage. These could be the result of a non-parasitic factor such as damage caused by a contact herbicide. A yellowing or browning of the veins also suggests the involvement of a non-parasitic factor such as the absorption of a herbicide by the roots, toxicity or mineral deficiency (Figure 2).

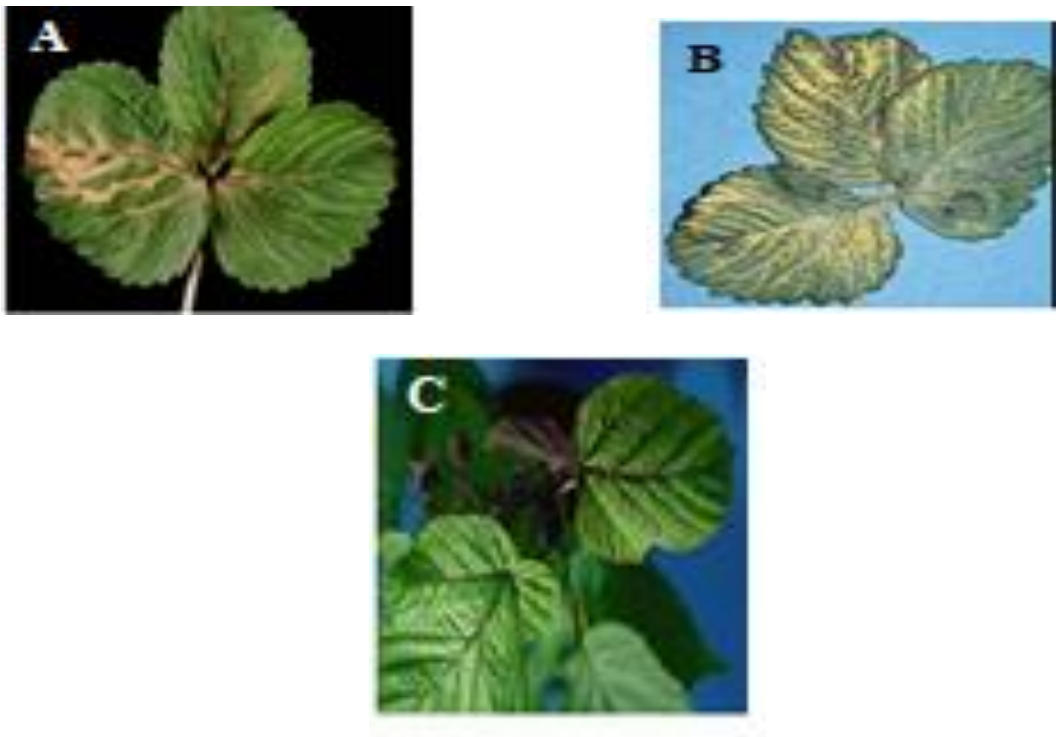

Figure 2 : (A) : Mineral potassium deficiency ; (B) : Phytotoxicity by a herbicide absorbed by the roots ; (C) : Blackening of the veins due to bacterial blight caused by the bacterium *Erwinia amylovora* (Lacroix, 2004)

Although it is true that a symmetrical pattern of symptoms on the affected parts of the plant suggests rather the involvement of a non-parasitic factor, we must remain cautious because some diseases caused by phytopathogenic organisms or pests will cause symmetrical symptoms.

Some parasitic diseases can lead to the development of spots with a symmetrical shape and distribution. Thus stains evenly distributed on leaves may suggest a non-parasitic problem but vigilance remains required in some cases...!

For parasitic problems, a greater variability of symptoms should be expected. Thus, the spots will tend to appear in different shapes and dimensions, with a light center and a dark margin, to be angular or targeted or accompanied by a yellow or aqueous halo. These characteristics are an indication of an evolutionary phenomenon, hence the presence of a parasitic agent.

I.1.1.2. Observing signs

In addition to the symptoms, it happens to directly observe the agent responsible for the disorder or certain signs of its passage. These signs may be present in cases of infection by a

fungus and bacteria or for damage caused by an insect or a mite. For some phytopathogenic organisms or pests, the sign is particularly visible and can be observed with the naked eye.

I.1.1.3. Host plant information

It is important to know which organisms can cause damage to various plant species. Although verticillium wilt (Verticillium), this disease is not significant in plants. In addition, not all cultivars are necessarily susceptible to the same pests, pathogens and abiotic factors. Finally, it is essential to know the origin of the plants. If the plants have been produced locally, it is possible that they are infected by the fungus responsible for anthracnose (Colletotrichum). It is important to know that this fungus does not survive under the climatic conditions of another country.

I.1.1.4. Date of onset of symptoms

The onset of symptoms can be linked to an event occurring at a specific time. Just think of the damage caused by climatic factors such as hail, strong winds or associated with a cultural practice such as the use of a pesticide, over-fertilization.

I.1.1.5. Problem development schema

The parameters presented under "pattern of disorder development" include the main elements to determine whether the cause of the problem is biotic or abiotic.

I.1.1.5.1. Problem distribution

Look at the vertical distribution, that is to say the location of symptoms on the plant. First, are the symptoms identical from one plant to another and secondly, are the symptoms on the affected part of the plant always located in the same place ? If the answer to these two questions is "yes", it is plausible to believe that the origin of the problem is not parasitic; it is logical to think about the involvement of an abiotic factor such as hail. The same goes for fruit damage. If the symptoms are present on the side of the external fruit at the row, it is possible that the damage is the result of insolation or hail.

Then, examine the horizontal distribution, that is, the distribution pattern of affected plants. If all symptomatic plants are located at the edge of the field or follow a symmetrical pattern, it is likely that the agent is non-parasitic (Figure 3). However, it is important to note that the

beginning of a pest infestation can be at the edge of the field while some parasitic diseases have a rectilinear pattern (on the row) as in the case of root rot (*Phytophthora*).

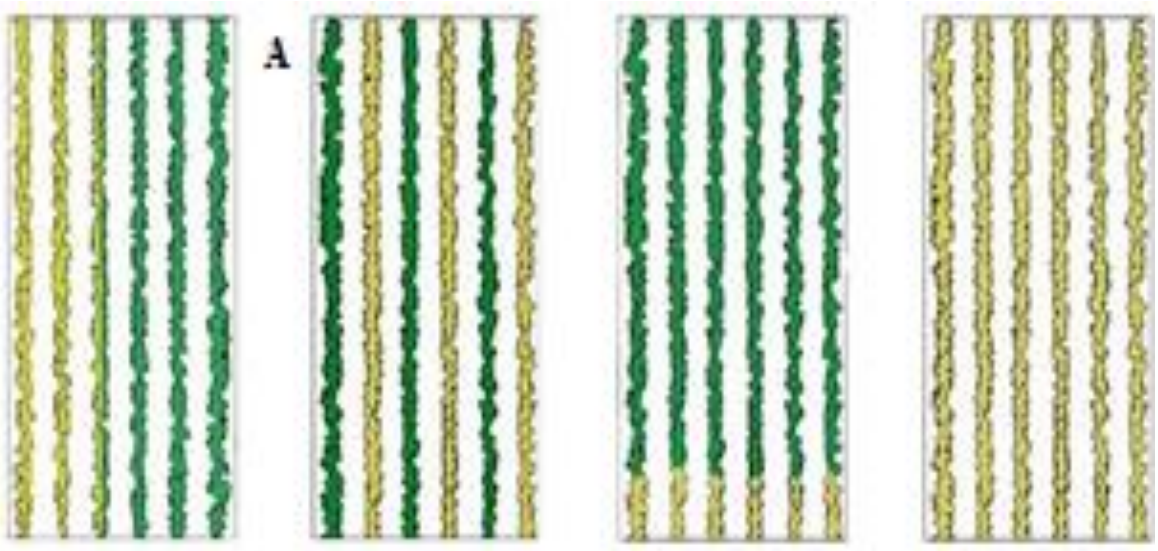

Figure 3 : A. Systemic horizontal distribution = abiotic (Lacroix, 2004)

If the affected plants are scattered or distributed in small groups (foci) (Figure 4), it is plausible to believe that a microorganism or pest is involved.

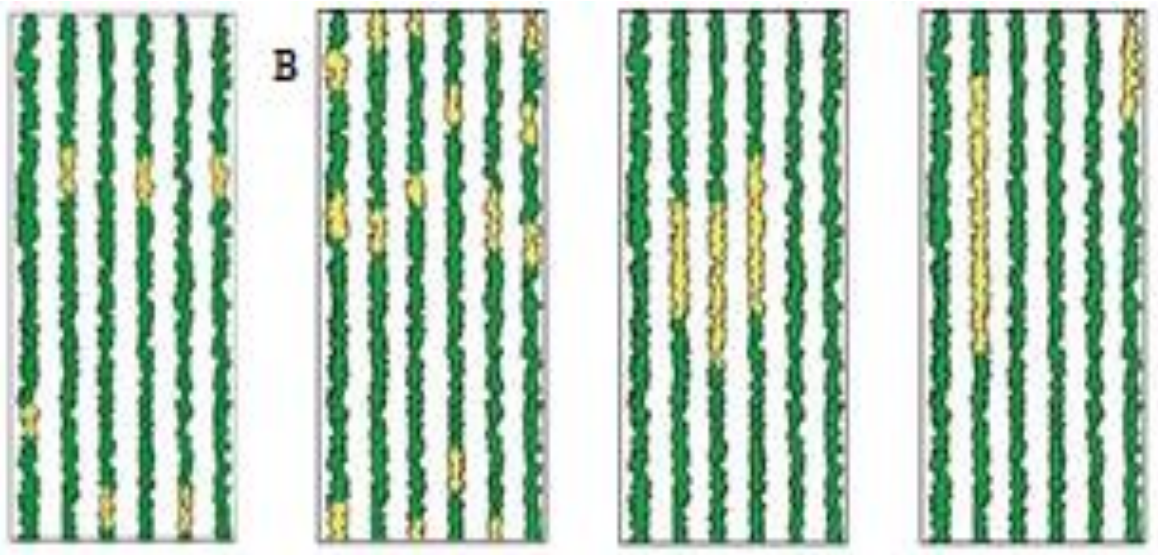

Figure 4 : B. non-systemic horizontal distribution = biotic (Lacroix, 2004)

I.1.1.5.2. Percentage of plants affected

Generally, when a high proportion of plants show a symptom, it is likely to think that the origin of the problem is non-parasitic. Conversely, if the proportion of affected plants is low to medium, a biotic agent may be suspected.

A. Damage appearance. A gradual onset of symptoms favors the involvement of a pathogen because it multiplies over time and produces damage that is intended to be progressive. The same goes for pests that reproduce quickly (mites, aphids) as well as for insects that are not very visible and cause damage to the roots, crown and stem. At first, the symptoms caused by these pests take some time to be visible to the eye, but their evolution is sometimes very rapid.

The affected plants should be compared to each other to check if the severity of symptoms varies from one plant to another, which can be a sign of the presence of an evolutionary problem caused by a pathogen or a pest. For abiotic factors, the symptoms usually appear suddenly and do not progress over time (hail, direct damage to the foliage by a herbicide, pollutants, mechanical injuries, drying of the stems by cold and dry winter winds, ...). However, we must remain cautious about this generalization because certain abiotic factors will induce progressive symptoms (e.g. mineral deficiency, herbicide in the soil, excess water, drought).

B. Affected species. If a symptom is found in several plant species, it is unlikely that a pathogen or pest is involved. It is therefore preferable to continue the process by considering mainly abiotic factors. It is important to observe whether weeds show the same symptoms. If yes, there may be a non-pest problem. We also need to check whether the problem is present in the fields of other producers in the region. If so, it is more than likely that the problem is related to an environmental factor.

I.1.1.6 .Climatic factors

Weather records are practical data. Extreme temperatures (heat and cold), rainfall (excess water or drought), relative humidity, winds (hot and dry winds that promote intense foliar transpiration, rain pushed by strong winds that cause mechanical injuries), the concentration of pollutants are major factors on which the diagnosis can be based. Climatic factors can be directly responsible for damage to a plant or promote the development of a parasitic disease or pest infestation.

I.1.1.7. Cultural practices

Information on cultural practices can be essential for establishing the diagnosis. The concentrations and application dates of fertilizers and pesticides, the type of soil (light soils favorable to nematodes, while poorly drained soils are favorable to soil fungi), drainage, soil pH and salinity, irrigation, crop rotation... may be involved in the onset of damage. Like climatic factors, cultural practices can predispose plants to infection by a pathogen (e.g., verticillium wilt in some plant species.

- **How is damage to crops discovered ?**

Some crop damage can be prevented by regular plant inspections.

- Inspect the entire field and not just a small part. To do this, walk in zigzag across the field.

- Stop off every now and then to inspect some plants. You may notice symptoms in the lower areas of the plant.

- Do not forget to inspect the lower surface of the leaves, or insects may appear.

- If you notice any damage, it may be useful to uproot the plant to see if the roots are also affected.

I.1. 2. Microscopic observation, cultures

In plant pathology, visual observation is often insufficient to determine the cause of a symptom and requires laboratory confirmation. This step represents a complementary sequence of the general diagnosis. The laboratory detection step can target a group of potential pathogens (example: fungi or bacteria), or a specific parasite (example: a bacterial genus or species).

I.1.2.1. Detection techniques

The detection techniques in a diagnosis are chosen according to the pathogen sought, the infected plant material.

I.1.2.1. 1. Isolation on culture medium

The isolation of phytopathogenic agents on agar culture medium remains the basic step often used in the diagnosis of bacterial and fungal diseases. Morphological identification (under a magnifying glass or microscope) completes the culture step to identify the pathogen, which

requires a good knowledge of the biology of the pathogen sought (Kox et al., 2007). This technique is qualitative and has the advantage of isolating the pathogen in pure culture, which can be used for later more advanced experiments, such as the koch postulate or in a possible scientific study (detailed study of the microorganism ...). This procedure has its limitations, as it is time-consuming (average duration of 5 to 10 days), and does not allow the detection of a large part of non-cultivable pathogens (mandatory parasites). In the case of mushrooms, the wet chamber technique is used to cultivate the parasite on its host (powdery mildew, downy...). The success of this process depends on the sample's freshness and sampling conditions (Schubert et al., 1988). In most cases, this method is completed by a microscopic observation to see the shape and characteristics of the parasite. If the results are insufficient to identify the parasite, confirmation by molecular biology is required.

I.1.2.2. Plant test

The Koch postulate is one of the methods used since the 18th century to confirm hypotheses in the diagnosis of a disease. It is used in plant diagnostics and can be summarized in four steps :

1) Isolate the pathogen from symptomatic tissue ;

2) multiply the parasite ;

3) inoculate a healthy plant with the parasite ;

4) re-isolate the parasite from the inoculated plant, which shows the same symptoms as the diseased parent plant. This procedure does not apply to unidentified parasites that are difficult to isolate, as is the case with certain viruses (Desvignes, 1999).

In addition to the Koch postulate, indexing is a fairly common technique used for the approval of new clones, by testing their susceptibility to certain diseases. It is also used in diagnosis to confirm symptoms caused by a given pathogen. It is applied to so-called indicator varieties that are sensitive to the disease studied. The susceptible plant is infected by rubbing a raw extract of the plant to be indexed, or with a solution of purified pathogen, infection is also possible by grafting a piece of tissue from the diseased plant. The test is placed in optimal conditions to develop symptoms. The appearance of these can be long, in the case of latent diseases (Devergne et al., 1982; Desvignes, 1999). Confusion is often possible when observing an anomaly on plants. Images A and B both show "white powder" on the upper

surface of the leaves. However, in the case of image A, it is the development of powdery mildew or "white", a fungus developing on the surface of the leaves. In the case of image B, it is an attack of aphids whose accumulation of exuviae (molt shells) gives a whitish appearance to the leaf. Two symptoms of similar appearance but two very different problems, which also involve very different means of struggle.

Presence of plant pathogens and pests by time of year. Overall, two periods are often more conducive to pest attacks on plants: from April to June and in October and November. However, the climatic conditions influence the attack periods which can therefore vary according to the seasons :

- Winter: Diseases and pests of storage preserves (foodstuffs, bulbs, rhizomes, etc.).

- Spring: Regrowth of emerging vegetation by insects, diseases, release of the first spores by fungi, transmission of the first viruses through vectors.

- Summer: Attacks of mites and insects (especially under cover), bacterial attacks, vascular fungal diseases and oomycetes.

- Autumn: Development of various fungi favored by the increase in humidity and the drop in temperatures.

A. Organs characteristic of the pathogen absent (biotrophs). The pathogen is only present in the injured tissues in a mycelial state, so it cannot be identified. Two types of techniques will be implemented in order to obtain the characteristic organs thereof :

- The damp room

The fructifications of the pathogen can be induced by placing the sample in conditions of humidity, heat and light favorable to this induction. But it is often essential to carry out a cultivation.

- The isolations

In general, except for the case of so-called mandatory parasites (powdery mildew, rust), the fungi present at the lesions are cultivated on artificial nutrient media. After disinfecting the attacked organ, a fragment of diseased tissue is taken and placed on a agar medium. The choice of culture medium depends on the nature of the organism to be isolated (those based on corn or oats are particularly suitable for Ascomycetes). In some cases, selective culture

media can be used, for example to prevent the development of contaminants (addition of antibiotic against bacteria) or to separate neighboring genera such as Ceratocystis EII. Exposure of the resulting crops to light and/or introduction of plant fragments into the medium promote sporulation. Sometimes, however, the culture remains sterile, requiring other identification techniques (Morelet, 1988).

I.2. Advanced techniques

I.2.1. ELISA and serological tests

More than 1,000 pathogens, bacteria and fungi can be detected using serological detection methods (Caruso et al. 2002) (Alarcon et al. 1990). The molecules of the pathogen can act as antigens by inducing the formation of antibodies. The serological antibody-binding reaction can be observed in vitro by placing a suspension containing the pathogen antigens and the antiserum. Antibody-antigen reactions are a powerful tool for the diagnosis of plant diseases. It is widely spread in phytopathology thanks to its specificity regarding a single antigenic exterminator (Martinelli et al., 2015).

I.2.1. 1. Elisa

Elisa is a method that has revolutionized the field of plant disease diagnosis worldwide. It was first used in the 1970s. Its principle is to detect the antibody-antigen interaction by labeling immunoglobulins with enzymes causing a color reaction. This technique has the advantage of being inexpensive. However, its sensitivity depends heavily on the organism and the state of the sample (Martinelli et al., 2015). Moreover, it is not susceptible to a wide range of pathogens (Fang and Ramasamy, 2015).

The use of an antibody specific to a bacterium or a phytopathogenic virus and an enzyme-conjugated antibody are the basic components for detecting and identifying these organisms. The enzymes usually attached to antibodies are alkaline phosphatase and peroxidase. The addition of a specific substrate to the enzyme used is then transformed into its presence and the products from this reaction show a yellow or orange coloration. Thus, the development of a stain in the wells of a polystyrene plate makes it possible to confirm the identification of the isolated bacterium or the presence of the desired virus in plant tissues. The ELISA test is carried out in a polystyrene plate generally containing 96 wells. It is important to prepare the covering antibody solution (capture antibody) just before use. The incubation of the plates is 4 hours at room temperature or 4 hours at 37oC or overnight at 4°C. After the incubation

period, the plate is washed 4 times with a washing buffer. It is most important to dry the plate well by hitting it vigorously on a paper towel. A residue of buffers and antibodies remaining in the wells may induce a false positive reaction. Negative and positive controls are prepared just before use.

The ELISA test provides a quantitative result that is visualized by the presence of staining in the wells where the pathogen is present. The more intense the staining, the greater the amount of bacterial cells or viral particles. The presence of staining in a well indicates that the enzyme-bound antibody has bound to the antigen, which had previously bound to the overlying antibody. In the presence of phosphatase, P-nitrophenyl phosphate is hydrolyzed to P-nitrophenol, which is a yellow compound. As for peroxidase, this enzyme degrades the peroxide (H_2O_2) and subsequently there is an oxidation of the ortho-phenylene diamine substrate (OPD) and the formation of an orange compound ((OPD)$2H_2$) (Devergne et al., 1982).

I.2.1.2. Immunoprecipitation

Immunoprecipitation is a rapid diagnostic technique for diseases. It is based on the formation of pathogen/antibody aggregates that can be observed when an infected plant extract is mixed with a specific antiserum. These aggregates are insoluble and visible to the naked eye or microscopy. The limitations of this method are mainly related to a lack of sensitivity. The use of an optical density reader allows a quantitative result to be obtained. For the optical density measurement, the filters used are 405 nm with P-nitrophenyl phosphate and 490 nm with ortho-phenylene diamine.

I.2.1.3. Immunofluorescence IF

Immunofluorescence is a method that combines microscopy and immunology. It is a method widely used in bacteriology. Immunofluorescence techniques involve coupling to the chosen antibody a fluorescent product that will make it possible to locate the site of the antigen-antibody reaction by observing these sections in ultraviolet light microscopy https://www.universalis.fr/encyclopedie/immunofluorescence/.

IF was previously used to detect an infection in onion culture caused by a fungus Botrytis cinerea (Dewey and Marshall, 1996). It has also been combined with other techniques such as FISH (Fluorescence In Situ Hybridization) for the detection of potato rot (Wullings et al.,

1998). This method is sensitive and specific but relatively long and not always easy to interpret.

I.2.2. PCR and sequencing (ITS, housekeeping genes)

Among the currently favored methods for diagnosing plant diseases, molecular biology is essential (Lopez et al., 2003). This technique is based on the analysis of nucleic acids from infected plants by polyacrylamide gel electrophoresis, or on the characterization of nucleic acids by molecular hybridization. The different indirect methods are :

I.2.2.1. PCR

PCR is based on the detection of specific nucleic acid sequences by molecular amplification. The use of this technique has improved the sensitivity of molecular diagnostic techniques. Real-time PCR platforms have been used for the rapid diagnosis of plant diseases based on bacterial, fungal and viral nucleic acids (Lievens et al., 2006) (Schaad and Frederick, 2002). Several types of PCR can be used:

A. Nested-PCR (N-PCR). This technique aims to improve the accuracy of results and increase sensitivity by performing a second molecular amplification.

B. Multiplex PCR. This technique involves placing several pairs of primers in the reaction medium to detect several pathogens at the same time.

C. Quantitative PCR. This technique involves detecting and quantifying the DNA of a given pathogen in real time. It is useful for large-scale diagnosis.

The PCR technique has high sensitivity and specificity. But, it depends heavily on the efficiency of the DNA extraction technique (Schaad and Frederick, 2002). In addition, the application of PCR for pathogen detection requires the design of a primer to initiate DNA replication, which could limit its practical application (Fang and Ramasamy, 2015).

I.2.2.2. Sequencing

Since the end of the 20th century, sequencing techniques have been developing and their costs decreasing, making them increasingly accessible in laboratories (Garrido-Cardenas et al., 2017). Thus, only a few decades after the discovery of the structure of double-helix DNA by James Watson and Francis Crick in 1953 (Watson and Crick, 2007), it is now possible to determine the sequence of nucleotides that make it up. Various sequencing methods have

been developed in recent years, but the Sanger principle developed in the 1970s is still widely used (Sanger et al., 1977).

I.2.2.2.1. Methodology and principle of Sanger sequencing

Several preliminary steps are necessary before using the sequencer. DNA extraction must first be carried out, followed by a PCR reaction using appropriate primers targeting the fragment(s) of interest. After purification of the amplicons obtained following the first PCR, another PCR: called "sequence" is carried out, which differs from conventional PCRs by the addition of dideoxynucleotides (ddNTPs) blocking DNA polymerase unlike deoxynucleotides (dNTPs). Once incorporated, the ddNTPs stop the elongation of the complementary strand because they do not have a hydroxyl group in position 3, making it impossible to incorporate a new nucleotide. In the reaction mixture, the enzyme randomly incorporates dNTPs or ddNTPs at a lower concentration, creating multiple fragments of different sizes depending on where the ddNTPs are inserted. Each ddNTPs is labeled with a fluorophore depending on whether it is Adenine, Thymine, Cytosine or Guanine. After the amplification step, the fragments will migrate by electrophoresis to be discriminated according to their molecular weight.

ddTTP = didésoxythymidine triphosphate ; ddCTP = didésoxycytidine triphosphate ;

ddATP = didéoxyadénosine triphosphate ; ddGTP = didésoxyguanosine triphosphate.

The DNA sequences obtained can be identified, thanks to constantly enriched databases, by comparison with reference sequences or sequences deposited by research laboratories. Computer programs such as BLAST (Basic Local Alignment Search Tool) allow the alignment of homologous sequences and the calculation of an identification score with respect to a sequence of interest.

A. Bibliographic analysis. It was necessary to develop new methods for identification and classification of fungi based on the DNA sequence. Since the 1980s and 1990s, universal primers have been sought to amplify certain fragments of interest and analyze the phylogenetic relationships between fungi. The challenge was to design primers for genes present in most or all of the genomes studied, with easy amplification and sequencing; the targeted genes must nevertheless contain non-coding variable regions that could undergo mutations, allowing the discrimination of different fungal species. The "panfungal" primers target highly conserved coding regions and generally consist of about twenty base pairs (bp);

they allow to amplify fragments of about 700 bp (compromise between a facilitated amplification and sufficient variability) comprising one or more variable regions.

Ribosomal DNA sequences quickly emerged as the sequences of choice : in 1982, Walker and Doolittle (Walker and Doolittle, 1982). Were the first to use the sequence of the ribosomal rRNA 5S gene to sequence certain basidiomycetes. Subsequently, White et al. in 1990 (White et al., 1988) marked a turning point in the history of fungal sequencing by developing universal fungal primers in the ITS region (Internal Transcribed Spacer), which are widely used today.

B. Ribosomal DNA and ITS region. Ribosomal genes repeat multiple copies in tandem within the fungal genome, which facilitates amplification; occurrences of this gene may differ between fungal species, but often exceed 100 copies. Regions encoding ribosomal RNAs are highly conserved and not very variable, so primers are often designed based on their sequences. Moreover, these rRNA precursors are separated by ITS variable regions, transcribed but non-coding, with high inter-species variability. The entire ITS region is about 600 bp and includes the ITS 1 and ITS 2 regions flanking the 5.8S rRNA coding region. In 5 of ITS 1 we find the 18S rRNA (SSU small-subunit) and in 3 of ITS 2 the 28S rRNA (LSU large-subunit). The 28S region contains two hypervariable D1 and D2 domains where several primers have also been designed.

On the one hand, mature 18S rRNAs will associate with other proteins to form the small ribosomal subunit 40S while, on the other hand, mature 5, 8S, 28S and 5S rRNAs (from another locus) will form a ribonucleoprotein complex with other proteins resulting in the large 60S subunit. The whole constituting the eukaryotic ribosome.

C. Fungal barcode. In 2003, the concept of genetic barcodes was introduced by Hebert et al. (2003). Assuming that at each position of the DNA sequence, the incorporation of one of four different nucleotides is possible, this generates for example for a sequence of only 15 nucleotides more than a billion possible "codes". Thus, assuming that mutations appear on variable regions, it is possible to think that each species has a "code" of its own constituting in some way its identification "barcode". The objective is to apply high-throughput sequencing technology to the large-scale screening of one or more genes defined as reference. The 650-bp region of the mitochondrial cytochrome c oxidase subunit 1 (CO1) is the animal barcode.

However, it has been excluded as a marker for the reign of fungi because of its difficulty to be amplified and its insufficient variability (Schoch et al., 2012).

The working teams formed examined, with regard to the fungal kingdom, various DNA regions to establish the best candidate for the fungal DNA barcode, namely : SSU (small subunit), LSU (low subunit), ITS (internal transcrib spacer), RPB1 (RNA polymerase II largest subunit), RPB2 (RNA polymerase II 2nd largest subunit) and MCM7 (Minichromosome maintenance complex component 7) (Raja et al., 2017). Ideally, the barcode region should have a constant and unique sequence for each species, with interspecific variation being much greater than intraspecific variation (Schoch et al., 2012). The difference between the genetic variability of isolates from the same species and the variability between different species is called "barcode gap" ; to allow proper differentiation and not lead to misidentification this gap must be positive (no overlap) between two close species (Meyer and Paulay, 2005).

The mycologists within the international CBOL initiative have finally defined the ITS region as the universal barcode region for fungi. One of the objectives of the international Barcode of Life (iBOL) was to create a database accessible to all in order to pool the work of each nation. In 2015, already 500,000 species (from the animal, plant and fungal kingdom) have been integrated into this database and this number continues to grow using new sequencing techniques. Their goal is to expand this number to 2 million species by 2026 (https://ibol.org/programs/program-overview/). Regarding the fungal kingdom, many ITS sequences have been added to the Barcode of Life Data System BOLD library (http://v4.boldsystems.org/), bringing the number of fungal species with a known ITS sequence to nearly 30,000 in 2019. (~16,000 Ascomycetes, ~12,000 Basidiomycetes, ~500 zygomycetes, etc.).

D. ITS region boundaries and secondary markers. Given the incredible diversity of the fungal kingdom, it is utopian to think that a single barcode region could identify each specimen as a species. Therefore, when the ITS sequence is not sufficient, second or even third line barcodes have been searched.

In the 2015 article by Stielow et al ., the authors, made up of members from several international teams, reviewed numerous candidates as secondary DNA barcodes for the ITS region (Stielow et al., 2015). They tested primers already known and used (Table 1) and

compared them with new primers created (targeting regions already studied or not) that amplify, among other things, TEF1-alpha (translation elongation facteur 1-alpha), TEF3 (facteur d'élongation de traduction 3), TOPI (ADN topoisomérase I), PGK (phosphoglycérate kinase), LNS2 (Lipin/Ned1/Smp2), etc.

Table 1 : Primers used to evaluate the performance of new primers designed in the article by Stielow et al., 2015

Locus	Primer	Oligo nucleotides (5'-3')	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	Ward & Adams (1998)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
	ITS1	TCC GTA GGT GAA CCT GCG G	White et al. (1990)
	ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	Gardes & Bruns (1993)
LSU	LROR	ACCCGCTGAACTTAAGC	Vilgalys & Hester (1990)
	LR5	TCCTGAGGGAAACTTCG	
TUB2	Btub2Fd	GTBCACCTYCARACCGGYCARTG	Woudenberg et al. (2009)
	Btub4Rd	CCRGAYTGRCCRAARACRAAGTTGTC	
	Bsens	ATCACWCACTCICTIGGTGGTGG	Lesage-Meessen et al. (2011)
	Brev	CATGAAGAARTGIAGACGIGGG	
ACT	ACT512f	ATGTGCAAGGCCGGTTTCG	Carbone & Kohn (1999)
	ACT783r	TACGAGTCCTTCTGGCCCAT	
	CA5R	GTGAACAATGGATGGACCAGATTCGTCG	Daniel et al. (2001), Daniel & Meyer (2003)
	CA14	AACTGGGATGACATGGAGAAGATCTGGC	
RPB2	fRPB2-5f	GAY GAY MGW GAT CAY TTY GG	Liu et al. (1999), Binder & Hibbett
	fRPB2-7cF	ATG GGY AAR CAA GCY ATG GG	
	fRPB2-7cR	CCC ATR GCT TGY TTR CCC AT	
TEF1α	EF1-983F	GCY CCY GGH CAY CGT GAY TTY AT	Rehner & Buckley (2005)
	EF1-1567R	ACH GTR CCR ATA CCA CCR ATC TT	

They evaluated the efficiency of amplification for different primers and this for different genera and families of fungi. First, it shows that the ITS region, already considered as a universal fungal barcode, has a success rate for PCR of about 92% (results very close to those obtained by Schoch et al. in 2012), closely followed by the LSU region with 88%. Moreover, among the newly developed primers on the TEF1-alpha region, the EF1-1018F/EF1-1620R pair also shows an interesting success rate which amounts to 82%. At this stage, other primer pairs seem promising targeting the gamma-actin (ACT) and beta-tubulin (TUB2) genes with respectively a success rate of 80% and 85%, but their efficiency is not homogeneous between different taxa and often low quality amplicons. In addition to having an efficient amplification and sequencing for different taxa, the potential secondary barcodes must have a significant discriminating power between different species, which is evaluated using the "barcode gap" mentioned above. The comparative analysis of the "barcode gap" shows,

among other things, a better resolution for the PGK, TOP1 and TEF1alpha regions than for ITS. In total, with more than 500 primer pairs evaluated on more than 1500 fungal species, it appears that the TEF1-alpha region, and more particularly the primers EF1-1018F/EF1-1620R, has currently the greatest potential to be defined as a secondary barcode.

E. Region TEF1-alpha. The TEF1-alpha gene consists of approximately 1600 bp with three introns. It codes for elongation factor 1-alpha, a ubiquitous protein required for protein synthesis. Several primers have been developed on this gene since 2005 by Rehner and Buckley (Rehner and Buckley, 2005) and improved during the decade, resulting in universal primers of higher fidelity.

F. Identification as a species. The ITS region is not ideal for some genera with many species including *Cladosporium* , *Penicillium* (Skouboe et al., 1999), *Fusarium* (O'Donnell and Cigelnik, 1997), T*richoderma* (Raja et al., 2017) and *Aspergillus* (Gonzalez et al., 2003) due to a lack of inter-species variability. In this case, identification to the rank of species may require the use of more specific primer pairs of the genera studied. Table 2 summarizes the different loci targeted and the corresponding primers that may be useful for fungal identification in general as well as the main candidates proposed for the identification of the genera mentioned above.

Table 2 : Summary table of primer pairs that can be used for fungal identification (Raja et al., 2017) ; (Skouboe et al., 1999)

	Locus	Amorces	Séquences 5'-3'	Réf.
Code-barres panfongique officiel	**ITS**	**ITS1** **ITS4**	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	(17)
Code-barres secondaire proposé	**TEF1-α**	**EF1-1018F** **EF1-1620R**	GAYTTCATCAAGAACATGAT GACGTTGAADCCRACRTTGTC	(24)
Alternatives	LSU	LROR LR5	ACCCGCTGAACTTAAGC TCCTGAGGGAAACTTCG	(30)
	RBP2	RPB2-5f RPB2-7cR	GAYGAYMGWGATCAYTTYGG CCCATRGCTTGYTTRCCCAT	(31)
Aspergillus	Calmoduline (CaM)	Cmd5 Cmd6	CCGAGTACAAGGARGCCTTC CCGATRGAGGTCATRACGTGG	(32)
	Beta-tubuline (BenA)	Bt2a Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	(33)
Fusarium	TEF1-α	ef1 ef2	ATGGGTAAGGA(A/G)GACAAGAC GGA(G/A)GTACCAGT(G/C)ATCATGTT	(34)
	TOP1	TOP_501-F TOP_501-R	TGTAAAACGACGGCCAGTACGAT CAGGAAACAGCTATGAC	(24,35)
	PGK	PGK_533-F PGK_533-R	GTYGAYTTCAAYGTYCC ACACCDGGDGGRCCGTTCCA	(24,35)
Penicillium	Beta-tubuline (BenA)	Bt2a Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	(33)
Trichoderma	TEF1-α	EF1-728F TEF1LLErev	CATCGAGAAGTTCGAGAAGG AACTTGCAGGCAATGTGG	(36,37)
Cladosporium	Approche multilocus ITS, Actine (ACT), TEF1-α +/- Calmoduline, Histone H3			(26)

Chapter II: Bacterial diagnosis

II.1. Isolation on selective media

II.1.1. Isolation and purification

Tissues collected at the healthy zone/sick zone boundary are disinfected on the surface and dilacerated in sterile water. After about twenty minutes of incubation, drops of this water are spread on a nutrient medium agar either with broad spectrum containing a fungistatic compound, or selective, if we have an idea about the desired parasite. After 1, 2 or 3 days of incubation at 30°C, we describe the colonies that have appeared on the medium and assess the importance of their presence. We then proceed to the purification by dilution of each type observed to obtain pure cultures, a second purification is often necessary. From the pure cultures obtained, some observations can be made : shape, color, rate of appearance of colonies, gram staining, highlighting of flagella, motility. A culture medium is said to be selective for a microbial species when only the specific nutrient requirements and development conditions of that species are met. By modifying different factors, it is therefore possible to direct a medium so that the growth of one species of bacteria is favored and others are inhibited. The parameters to be modulated are incubation temperature, pH, nutrient source (carbon, nitrogen...), presence of antibiotics (actidione, bacitracin...) or antiseptics (berberine, sodium selenite...). In this medium, cells generally find the elements necessary for their growth, such as a source of carbon and energy (glucose, lactose, fatty acids, etc.), a source of nitrogen (ammonium (NH_4^+), nitrate (NO_3^-), amino acids, peptone, etc.), growth factors (Vitamins, essential amino acids, NAD+ (Nicotinamide adenine dinucleotide and blood as growth factors for demanding bacteria) and Hems, which are components of the cytochromes necessary for electron transport chains.) and some trace elements (Iron (Fe), zinc (Zn), copper (Cu), cobalt (Co), manganese (Mn), etc. (Tankeshwar, 2021).

However, it is necessary to add additional components or nutrients to the carrier when growing particular microorganisms. In addition, it is necessary to adapt the pH of the medium accordingly. Culture media can be classified in three ways: classification based on consistency (solid, semi-solid, liquid, biphasic media), classification based on chemical composition (complex media, synthetic media, semi-synthetic media), and classification according to the function of the medium (basal media, enriched media, selective media, enrichment media) (Tankeshwar, 2021).

II.1.2. Chromogenic culture media

These are selective and differential culture media that contain chromogenic substrates that react with the specific enzymes produced by certain microorganisms, resulting in a characteristic color change. In microbiology, these media are extremely beneficial for rapid and accurate detection of bacteria (Prinzi, 2020). When bacteria grow on these media, chromogenic substrates are metabolized by particular bacterial enzymes, resulting in the production of colored pigments.

The color that develops may be specific to certain species or groups of microorganisms, which allows for visual identification of bacterial colonies. These environments are used in various sectors to check for the presence of a germ of interest. Thanks to their technology, the detection of germs becomes more reliable and more precise, which avoids certain errors. The substrate used is carefully selected to ensure optimal specificity (Prinzi, 2020).

A. Macroscopic methods. Refers to the visual observation and description of an object, sample or structure at a scale visible to the naked eye or with simple optical instruments such as low-magnification microscopes. The macroscopic study of a sample in microbiology, for example, may involve observing its shape, color, size and any other characteristic visible to the naked eye. This technique is frequently used to study whole organs, tissues or organisms (Michael et al., 2015).

B. Highlighting of physiological and biochemical characteristics. It is essential to carry out various tests (aerobic or anaerobic use of glucose, growth on different carbon substrates, ability B to degrade certain macromolecules, sensitivity to NaCl, maximum temperature allowing growth etc.). It is necessary to choose a range of tests that will allow him to determine the genus and species of the pathogenic bacteria present in the tissues.

II. 2. Biochemical tests (API, Biolog). These tests allow the identification of morphological and metabolic characteristics, including: shape, wall structure, presence of flagella, glucose utilization pathways (fermentative or oxidative), respiratory type (strict aerobic, optional anaerobic...), the carbon and nitrogen sources used and the characteristic enzymatic systems (oxidase, catalase, nitrate reductase...). In routine work, five tests are sufficient to identify with certainty the bacterium studied, but it is necessary first to have an idea of its nature to carry out the appropriate tests. This difficulty has been overcome by the appearance of miniaturized biochemical test galleries.

II.2.1. API 20E

The API 20E gallery (bioMérieux) is a miniaturized version of the classic biochemical tests intended for the identification of Enterobacteriaceae (Gram-negative bacteria and facultative anaerobes), including *Erwinia*. This system includes 23 biochemical tests. Dehydrated substrates are contained in microtubes. These substrates are dissolved by adding a suspension of the bacteria to be identified. Following an incubation period (24 hours at 30°C), allowing the bacteria to react with substrates, the various reactions are recorded in order to determine the 7-digit identification code.

A. API 20 E1. The manufacturer's instructions should be followed to prepare the suspension and inoculate the gallery. Incubate the gallery at 25–26 oC. After 48 hours, a culture of E. amylovora should typically give the following results: negative for lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate consumption (CIT), H2S production (SH2), urease (URE), tryptophan deaminase (TDA), indole production (IND) and rhamnose oxidation (RHA), and positive for sucrose oxidation test (SAC). Other tests may vary depending on the strain, according to Donat et al. (2007).

B. API 50 CH1. Prepare a suspension of optical density 1.0 (at 600 nm) in PBS. Add one millilitre of this suspension to 20 ml of the Ayers medium (NH4H2PO4, 1 gram; KCl, 0.2 grams; MgSO4, 0.2 grams; bromothymol blue, 0.2%, 75 ml; distilled water, 1 liter; pH 7; sterilized at 120 oC for 20 minutes) (Ayers et al., 1919). The inoculation of the gallery should be carried out according to the manufacturer's instructions. Incubate the gallery at 25–26 oC under aerobic conditions. When a well turns yellow, it means that the body is consuming the carbohydrate in question. After 72 hours, a culture of *E. amylovora* should typically be positive for these carbohydrates: L-arabinose, ribose, D-glucose, D-fructose, mannitol, sorbitol, N-acetylglucosamine, sucrose, trehalose, and β-gentiobiose. Other sugars are not consumed by E. amylovora under the conditions of this test, but Donat et al. (2007) indicate that some strains may use glycerol and D-fucose.

II.2.2. Biolog

For the diagnosis of bacterial diseases, the first step is to isolate the bacteria from plant tissues. The isolations are carried out on culture media coated with either B de King for the *Pseudomonas* (King, E.O., M.K. Ward and D.E Raney. 1954), MS for the *Pectobacterium* (Miller-Schroth medium) and LPGA (Yeast Peptone Glucose Agar) for the other bacterial

genera. After soaking plant tissues in a saline solution (NaCl 0.85%) for 30 minutes, the suspension is spread by exhaustion on the culture media. The incubation is done at 26°C and it is generally from 48 to 72 hours in order to obtain colonies of 1 to 2 mm in diameter (Breton et al., 2014). The *Xanthomonas* colonies on the LPGA medium have a pale yellow to lemon yellow coloration. They are slightly convex, glossy, with a well-defined margin. *Pseudomonas* colonies on King's B medium are whitish to cream in color with or without the presence of fluorescence when colonies are subjected to ultraviolet light.

- The identification of aerobic Gram-negative and positive bacteria by the Biolog system is possible thanks to the use of GEN III plates. They make it possible to simultaneously check the metabolic reaction of bacteria against 94 phenotypic reactions (71 carbon sources and 23 tests of sensitivity to chemical inhibitors). Following the inoculation of a GEN III plaque with an unknown bacterium, a metabolic profile is obtained.

- Ioculation of the Biolog plate. Bacterial colonies isolated from plant tissues with the morphological characteristics of the desired bacteria are put in pure culture on TSA medium (Tryptic Soy Agar). The first step is the preparation of a bacterial suspension in an inoculation liquid tube (IF) type A (Green). The bacterial suspension must have a transmittance of 90 to 98%. The turbidity of the bacterial suspension is measured by an electrophotometer or a turbidimeter. Inoculation of the GEN III plate is carried out by depositing 100 µl of the bacterial suspension in each well. Incubation is carried out in a humid chamber at 30°C for a period of 24 to 48 hours.

- Reading of the Biolog plate. The identification of bacteria by the Biolog system is carried out using an automatic plate reader combined with a computer database containing the reference bacterial profile. Well A1 must be colorless. The use of substrates or resistance to chemical inhibitors by the bacteria results in the appearance in wells of a purple color. This purple coloration is produced by the reduction of the indicator, tetrazolium, into a colored compound, formazan. In the absence of use of the substrate or if the bacterium is sensitive to the product, the well remains uncoloured.

- Interpretation of the results. Following the reading of the results of the biochemical tests obtained for the unknown bacterium, the Biolog identification system compares this metabolic profile with those of the reference bacteria in its database. Identification is therefore

carried out by matching the profile of the bacterium to be identified with that of the bacterial species showing the most similarity. The identification is mainly based on two values.

- Similarity is an index reflecting the degree of matching between the metabolic profile of the bacteria to be identified and that of the bacteria proposed by the Biolog system. The closer the value gets to 1, the better the identification is considered.

- The distance indicates the number of wells whose results are different between the unknown bacteria and those proposed by the Biolog system. The closer this difference is to zero, the better identification is considered.

II.3. Molecular tools (multiplex PCR)

Molecular biology is currently an essential tool in the detection of parasites in phytopathology. PCR is widely used, it is applicable to all organisms and works even on samples of small amount of material. This technique is also appreciated for its speed and the ability to analyze several samples at once (Kaluzna et al., 2010). One of the limitations of this technique is the presence of inhibitors in the sample to be analyzed, such as polyphenols, which requires optimization during DNA extraction from the sample (López et al., 2010).

In the majority of cases, PCR (or RT PCR in the case of RNA viruses) is insufficient to establish a complete diagnosis, so other techniques are used, such as high-throughput sequencing (HTS), followed by bioinformatics analysis (Caporaso et al., 2011). This last technique offers the possibility to determine exactly the micro-organism, and allows to refine the analysis to identify the species or subspecies (Šafárová et al., 2013). In bacterial diagnosis, RFLP and MLST analysis of 16s DNA is sometimes necessary to complete the PCR results, for example in order to determine the species or pathovar. This information is useful for implementing a treatment that specifically targets the identified pathogen. This approach is generally adopted in the context of research or research and development to improve plant protection products (fungicides, bactericides...) (Gonzalez et al., 2003; Leroux, 2003).

II.3.1. Multiplex PCR

The multiplex PCR protocol aims to amplify multiple amplicons simultaneously using at least three primers per PCR reaction. The PCR products will then only be able to compete with polymerase, dNTPs and possibly the DNA marker. Different types of DNA recognized by the same primer pair can also be amplified. PCR can be multiplexed at the endpoint (PCR

products are generally distinguished by their size or the presence of a restriction site), or in real-time for several different targets by measuring the melting temperature of PCR amplicons with conventional double-stranded DNA dyes such as SYBR Green. It has many qualitative applications such as the detection of viral strains, mutations, etc...

Before performing your multiplex PCR, you must indicate the target DNA sequences to be amplified in a database such as GenBank, then design specific primers and check their compatibility to be mixed together in the same PCR reaction (Bustin, 2000) .

Once the target sequences are selected, you can design specific primers. The choice of primers is essential for successful multiplex PCR. The rules applied to the design of primers for simple PCR must be respected in the case of multiplex PCR. Here are some of the most important :

- The size of the primers is often longer between 22 and 25 nucleotides.

- The GC percentage of the primers should ideally be between 40 and 60%.

- It is recommended to avoid repetitions in 3' GC of each primer.

- Online software such as PrimerPlex and Prime3 Plus (freely available) are suitable tools for primer design.

II.3.1.1. Check the compatibility of the primers

Once primer sequences have been defined, their compatibility must be checked. Check the following parameters so that they work correctly in multiplex PCR (Debode et al., 2017) :

- Les Tm (melting temperature)

The differences between the Tm of the primers must be reduced to as close as possible so that they can function at the same hybridization temperature. It is advisable to design the primers with Tm around 60-65°C to work with a 2-step PCR program.

A. The structures of sequences

Not all primers must contain :

- Stable secondary structures such as rod-loop structures

- Complementary regions of more than 3 nucleotides in 3'.

The specificity of the primers must be strictly verified to avoid any non-specific amplification. Complementarity searches between the primers, as well as homology searches of each primer with the target DNA sequences are also to be carried out. Online software such as BLAST, ClustalW, NetPrime are available for free access to these checks and to validate the primer sequences.

B. The lengths of the amplicons. If you separate PCR products by electrophoresis, you must choose primers so that the sizes of the sequences to be amplified are sufficiently distinct from each other to be able to identify them on gel and separate them. The minimum gap between each PCR fragment depends on:

- The size of the longest fragment to be amplified in a multiplex PCR reaction.

- Of the gel type: a polyacrylamide gel is generally more resolving than an agarose gel.

- From the electrophoresis system: gel or capillary.

For example, if the longest fragment size is 1000 bp and you use an agarose gel, there should be at least 100 bp difference. Check the resolution of your separation system before setting the sizes of the amplicons.

II.3.1.2. PCR validation

Each primer pair must be previously validated by simplex PCR using a specific PCR enzyme, as multiplex PCR amplifies several targets in a single reaction and the specificity of each amplification is paramount. A hot start PCR enzyme is recommended, as it prevents non-specific amplifications by inactivating the enzyme activity at low temperatures, where non-specific hybridizations preferentially occur.

II.3.1.2.1. Validation by simplex PCR

Each pair of primers must be individually tested and validated in simplex PCR before multiplexing. Prepare one reaction mixture per target in the same way and follow the same PCR program for each mixture. Each simplex PCR must amplify a single sequence to the expected size. If necessary, optimize the temperature and/or hybridization duration, and/or elongation duration. Optimize the conditions so that all simple PCRs work optimally at the same hybridization temperature. If these adjustments are not sufficient to obtain optimal

amplifications, it is advisable to design new primers. We do not recommend optimizing reaction mixtures at this stage.

A . The optimizations of multiplex PCR. Once all the primer pairs are working individually in simplex PCR, you can start multiplex PCR tests. Use the longest hybridization and extension time and hybridization temperature validated in simplex PCR.

B. The reaction mixture. It is advisable to mix the primer pairs in small groups. Start with tests with 2, 3 or 4 primer pairs, then add 1 or 2 additional primer pairs until all primers are included. Mix all the reagents under conditions validated in simplex PCR. The primers to be multiplexed are equal molars at the start.

C. The programming of PCR cycles. The 2-step PCR is more specific and recommended for multiplex PCR rather than 3-step PCR.

The duration of hybridization and elongation is often longer for multiplex PCR due to a larger number of primers. Depending on the hot-starting enzyme chosen, the initial denaturation time can vary from 1 to 2 minutes up to 15 minutes. This is related to the method used to inactivate the enzyme at low temperatures. It is important to follow the manufacturer's instructions to fully benefit from the enzyme's performance.

If the amplifications of all targets are weak : increase the extension duration, adjust the amount of enzyme and/or DNA template. The maximum volume of added enzyme must not exceed 10% of the reaction volume. The hybridization temperature can also be adjusted downward, in increments of 2°C.

- If target amplifications are not homogeneous : increase the concentration of primers for weakly amplified targets.

- If non-specific amplifications are obtained, increase the step hybridization temperature by 2°C; increase the magnesium concentration.

- In some cases, the addition of PCR additives such as BSA, DMSO can be useful.

It is recommended to modify only one parameter at a time (Rajtak et al., 2011; Li et al., 2017).

Chapter III : Viral diagnosis

III.1. Tests on indicator plants

The host family includes test or indicator plants whose responses characterize a particular virus. A good host range should allow the testing and study of virus multiplication for the determination of its biological properties in raw juice. A good test plant should be one that develops distinct symptoms early after inoculation, and rapid growth. For mechanical inoculations, the local lesion plant is by far the best test plant. As an example of host range, we will give that of the tobacco mosaic virus (TMV) which we identified on samples of tomatoes.

The transmissibility trait is a fundamental property of viruses and other biological beings that cause disease. The pathologist is concerned with the transmissibility of viruses both from a practical point of view by trying to avoid or limit natural transmission, and from an experimental point of view because most virological research depends on the transmission of the virus under controlled conditions.

- Transmission by graft. Grafting is the method of virus transmission that can be used universally, requiring only that the virus determine a systemic infection in plants where it is being transmitted. Originally, grafting was an ancient horticultural practice.

After inoculation of healthy tomato plants with diseased cuttings. They could observe symptoms of deformity, leaf curling and spire reversal in the feet of tomatoes trimmed at the beginning of production. Such symptoms that affect the plot by more than 90% can be due to a viral disease. The transmission of viruses by graft proves difficult and even impossible between family plants distant by organic incompatibility.

- The possibility of virus transmission through the seeds of diseased plants has been recognized since the first virology studies. Nowadays, a large number of viruses transmitted by the seed are known, including cucumber mosaic virus, bean virus 1, lettuce mosaic... The rate of transmission through the seed is extremely variable (10,000 up to 75%.

- A large number of viruses (about half) are transmitted by insects. These insects belong to various orders, the most important being the Homopterans (leafhoppers and aphids). Most of these insect vectors are stingers (Thysanoptera, Heteroptera and Homoptera). We also identify vectors among the grinding insects : *Coleoptera, Locust* and *Wildfowl*.

Some viruses are transmitted by living beings in the soil, such as nematodes or lower fungi (Olpidiaceae, Synchytriaceae and Plasmodiophoraceae). Examples :

- The ringspot viruses of fruit trees and vines are transmitted by nematodes belonging to the genus *Xiphinema*. The nematode *Trichodorus* transmits Tobacco Rattle virus and Tomato black-sing virus.

Particular strain of the fungus Olpidium brassicae is the mandatory vector of the Big vein of lettuce and the stunt (dwarfism) of tobacco. *Polymyxa graminis* is a vector of wheat virus and peanut stunting. Viruses can be transmitted from one generation to the next through vegetative propagation organs: bulbs, rhizomes, tubers, stolons and cuttings.

III.1.1. Symptomatology

The activity of a virus in a plant can lead to the appearance of particular localized or systemic symptoms.

- Localized symptoms: they are characterized by a hypersensitivity reaction of the host plant by limiting the viral infection at the inoculated site. The bean virus we studied induced necrotic local lesions on Phaseolus mungo and chlorotic local lesions on *Cucumis sativus*.

- Systemic symptoms: they first appear on the youngest organs or those inoculated, then spread throughout the plant. Mosaics, variegations, spottings, chloroses, necrosis, foliar deformations and stunting can be distinguished among others.

III.1.2. The properties of virosed plants

These properties, also called "biochemical tests", are methods for detecting and characterizing well-known viral diseases. Biochemical tests in Virology are based on the fact that various modifications appear in plants during viral infection. These modifications consist either in the appearance of new substances or in the stimulation of the production of normally present and non-pathogenic substances. The methods considered are various: colorimetry, spectrophotometry, chromatography, electrophoresis, precipitation...

Some viruses, all mechanically transmissible, and referred to as non-persistent viruses, can be inoculated by aphids as soon as they leave the diseased plant acting as a source of virus and then inactivate very quickly, within minutes or tens of minutes. Tobacco mosaic virus and potato virus X) show that the aphid is not a purely mechanical vector agent.

Other viruses called persistent viruses remain infectious for a very long time in the body of the insect vector, sometimes throughout its life. Most of these viruses are not mechanically transmissible (Bawden, 1950). They have a latency period in the organism of the insect vector. Indeed, it only inoculates them several hours, sometimes several days after leaving the plant that is a source of the virus. This latency period certainly does not have the same cause in all cases. In certain viruses transmitted by leafhoppers (Aster yellows virus, yellow dwarf virus of the potato, club-leaf of the clover, stunt disease of the rice), it is accompanied by a multiplication of the virus in the insect's organism.

III.1.3. Techniques used to characterize viruses

Different methods are used in virology to characterize viruses : spectrophotometry is the characteristic absorption of viruses in the ultra-violet ; the sedimentation rate obtained by accelerated centrifugation and which provides information on the sedimentation constant at a given temperature, the diffusion, which is the transfer rate for particles in water at 20°C and which is expressed as the diffusion constant, the measurement of viscosity for very pure suspensions of viruses, electron microscopy which allows direct observation of particles, Electro-phoretic mobility and electrophoresis is a method based on the principle that certain substances move when subjected to an electric field. The field leads to the separation of the various constituents according to their respective migration rate (Fauquet and Thouvenel, 1977).

III. 2. Electron microscopy

The electron microscope is a tool that allows to visualize extremely small objects. For this reason, it has been used extensively to characterize and identify viruses. Designed in the 1930s, notably by Ernst Ruska who won a Nobel Prize for it in 1986, the transmission electron microscope (tEM) generates an electron beam from a cathode subjected to a very high voltage. This electron beam is directed onto a sample that it passes through to reveal the image on a fluorescent screen, a photographic plate or more recently on a CCD camera which can then reveal the image in real time on a monitor. Other microscopes such as the scanning electron microscope (sEM) or the atomic force microscope (aFM) also make it possible to visualize the structure or details of viral particles.

Electron microscopy is able to provide the following information on viral particles : particle size, shape, internal and external structure, aggregation or crystallization states, quantitative

analyses of virus suspensions. A commonly used method for the rapid detection of viruses in diseased plants is the dip method or "dipping". It consists of depositing a drop of raw juice on a carbonaceous electron microscopy grid. Then a negative staining is carried out with Uracycle acetate or Uracycle formate or even phosphotunstate. It should be noted, however, that the dip method is applicable mainly to viruses with long particles, because the unpurified juices of diseased plants contain normal cell constituents which are essentially spherical in shape and in the same size range as round viruses.

The technique makes it possible to identify the virus based on its structure and size. Its main advantage is the speed of implementation. It can be combined advantageously with immunological techniques: they then spoke of immunological decoration (the viral particle is surrounded by immunoglobulins which give it a denser appearance), immunosorbent electron microscopy (IsEM) when viral particles are adsorbed using immunoglobulins, somewhat like an ELISA.

The isolation and purification of the virus will allow the preparation of samples to be examined under an electron microscope. A drop of the purified suspension is deposited on a grid, previously covered with a film of carbon-based collodion. The viruses are then stained negatively (the dye penetrates the virus), with for example 0.5% Uracycle acetate. The grids can then be observed directly under an electron microscope. www.virologie-uclouvain.be

III.3. RT-PCR and high-throughput sequencing

According to Walter (1988); Semal (1993) and Amedjkouh (2004), the specific identification of a viral infection involves a series of techniques leading to the identification of the virus, namely:

- Methods of virus detection by direct observation of symptoms

- (Symptomatological detection).

- Serological detection (ELISA test).

- Molecular biology method (RT-PCR).

III.3.1. Molecular biology technique (RT-PCR)

The reverse transcriptase polymerase chain reaction (RT-PCR) is a variant of PCR or polymerase chain reaction. Both techniques rely on the same process, but RT-PCR provides

an additional step is reverse transcription of RNA into DNA or RT, which is required for amplification of genetic material in vitro. Highly sensitive and specialized real-time RT-PCR technology allows for reliable diagnosis (Jawerth, 2020).

- The principle of PCR

This involves selective amplification of target fragments using a specific primer pair selected from well-defined sequences, viral genomic DNA and thermostable enzyme (Taq polymerase) (Saiki et al. 1988; Selmi, 2018).

III.3.1.1 Implementation of a PCR test

- A reaction medium for PCR contains:

- The initial target DNA, as appropriate, is viral DNA or complementary DNA synthesized in the preliminary steps.

- Two primers that hybridize on the sense fragment to each other on the antisense fragment and that delimit the amplified region.

- Deoxynucleotides triphosphate (dNTP).

- Magnesium (Mg2+) (enzyme cofactor).

- Taq polyemase

Protocol for virus detection by reverse transcription-PCR (Arezki, 2016):

A. Nucleic acid extraction. The nucleic acids were extracted according to the protocol developed by Foissac et al. (2001), for example from cortical phloem dehulled using a scalpel on vine cuttings.

B. Quantification and purity control of extracted DNA. The yield and purity of extracted DNA were determined by measuring the absorbance under UV light at 260, 230, and 280 nm using a spectrophotometer (Rahali, 2020).

C. Amplification of complementary DNA (cDNA). After obtaining complementary DNA (Lehad, 2012): Choose the DNA primers used for RT-PCR: allow to identify virus types (Lehad, 2012).

Consequently, this technique can double the amount of DNA in selected regions according to three main steps (Rahali, 2020):

- Denaturation step.

- Hybridization step.

- Polymerization or extension step.

D. Preparation of samples for PCR. Add each cDNA sample to the reaction medium with forward and reverse primers as well as Taq polymerase and distilled water. The samples were then placed in a thermal cycler (Lehad, 2012).

E. Analysis of PCR products. After amplification, an electrophoretic separation was performed on an agarose gel. The presence of DNA can be observed using a DNA intercalator, such as ethidium bromide, which fluoresces under UV light.

With optimal techniques for extracting total RNA. RT-PCR followed by repeat of the ethidium bromide amplification products still detects all three viruses from woody tissue throughout the year (Kummert et al., 1998). From the perspective of routine use of PCR, however, problems remain given (i) the cumbersome sample preparation protocols. (ii) the risks of accidental sample contamination and (iii) the insufficient sensitivity and specificity of the amplification products detection step. The simplification of RT-PCR protocols and the increase in their sensitivity make it possible to consider routine use of molecular detection tests.

F. Reducing the risk of contamination from handling. Recent kits allowed us to perform both RT-PCR reactions (transcription of RNA and amplification of DNA sequences) sequentially in the same tube, thus reducing manipulation and contamination risks of samples (Mallet et al., 1995).

Chapter IV : Animal pests

IV.1. Identification keys for insects/nematodes

IV.1.1. Insect identification key

This determination key is based on morphological characters called "identification criteria". It allows us to determine the adults of 15 orders of insects easily observable. The most common representatives of each order complete the identification. Aquatic species and the majority of soil inhabitants are not taken into account (Mignon et al., 2016) .

Once on the field, start by capturing one or more small animals. Be careful how to proceed. "The four insect seasons", information on how to behave and capture methods. Once the animal is caught, carefully examine it. Take the time to study it and discover it :

• Does he have wings ?

• What does his paws look like ?

• What form does his body take ?

• Does he really only have a pair of wings ?

Then identifies its order using the determination key.

IV.1.1.1. Use of the determination key

1. Choose the small animal whose order you want to determine.

2. Starts the determination at step 1 of the key.

3. Read the first two identification criteria from step 1 and identify which one corresponds to your insect.

4. After choosing the correct criterion, look at the end of the line in the right-hand column.

5. If there is a number, it’s the next step you need to take to continue determining your insect. Then continue the determination.

6. If there is no number, you will find a word. It is the name of the order to which your insect belongs (Figure 5, 6, 7, 8, 9, 10, 11, 12 et 13). In addition to the order, some examples of insects among the best known of this order are noted (Pro Natura, 2021 ; Mignon et al., 2016 ; Kouakou et al., 2016; LaSalle, 1987 ; Hayat, 1983 ; Woolley, 1988).

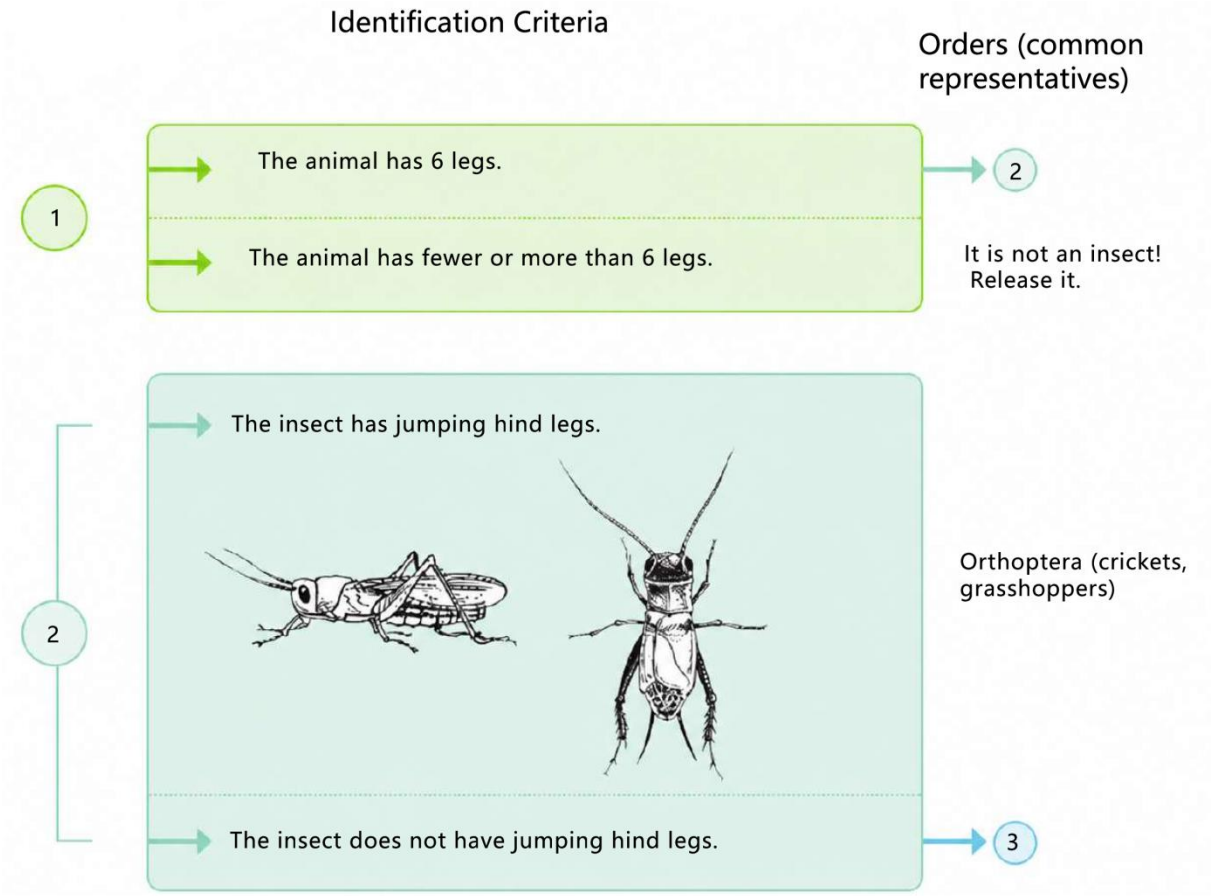

Figure 5: Stages (1 and 2), the insect has jumping hind legs (Orthoptera). (Mignon et al., 2016) ; (Pro Natura 2021)

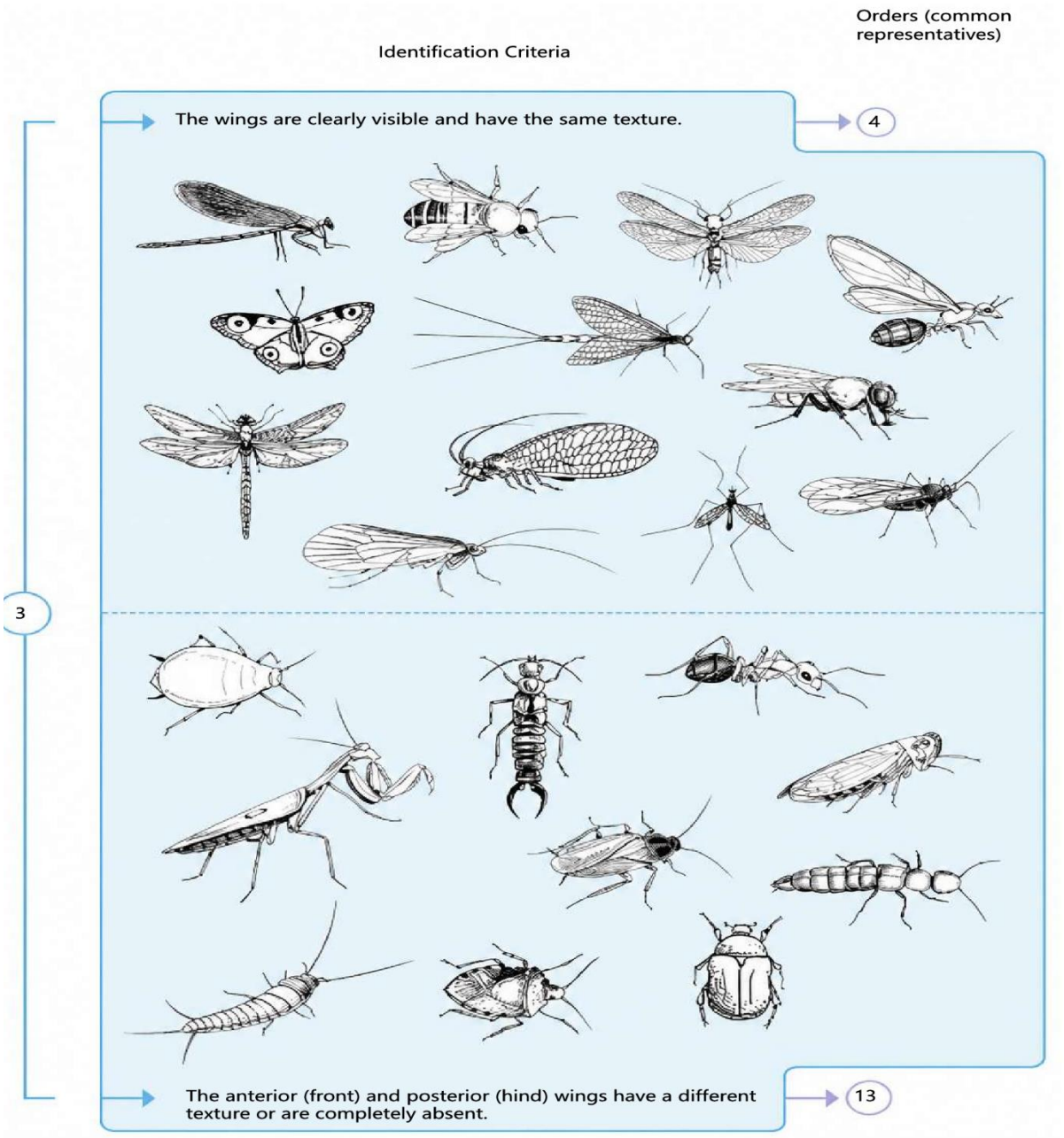

Figure 6 : Step 3 including insects with visible wings and the same texture, insects with anterior and posterior wings and others with different texture. (Kouakou et al., 2016) ; (Pro Natura, 2021)

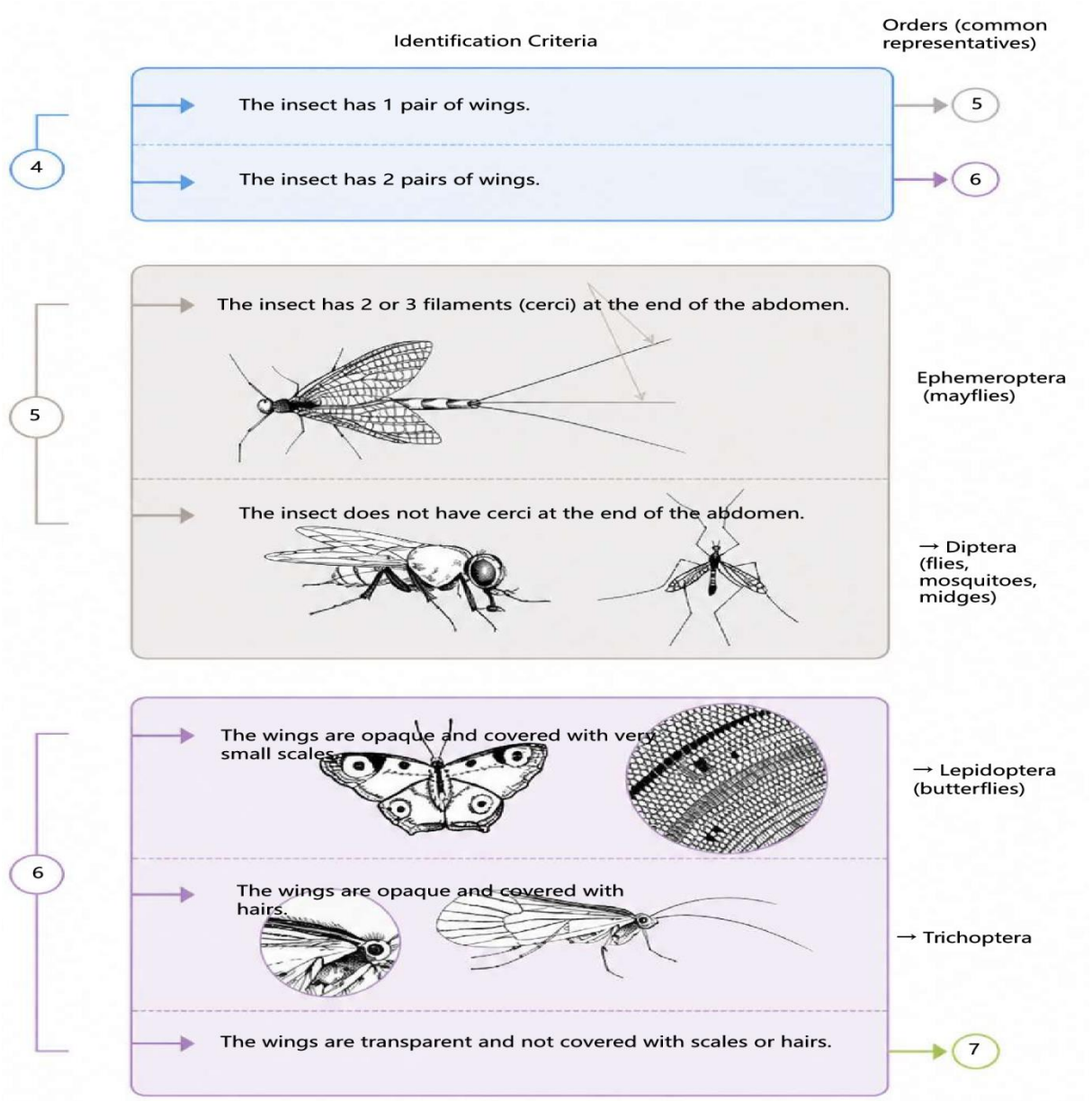

Figure 7 : Stages (4, 5 and 6) include insects with opaque wings covered by scales, with hair wings, and transparent wings. (Mignon et al., 2016) ; (Pro Natura, 2021)

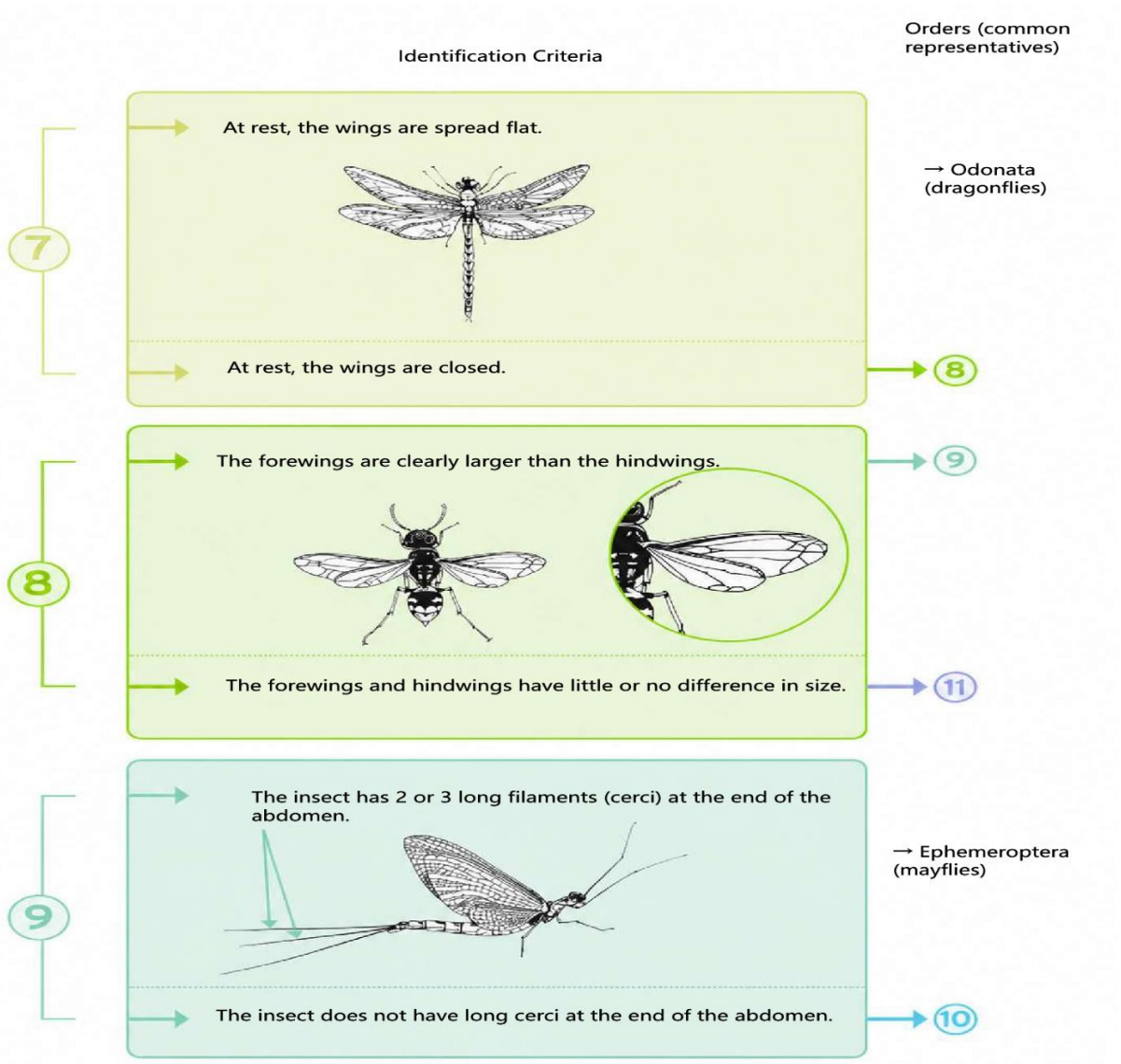

Figure 8 : Stages (7, 8 and 9) include insects with open wings, larger forewings and insects with cerci. (Mignon et al., 2016) ; (Pro Natura, 2021)

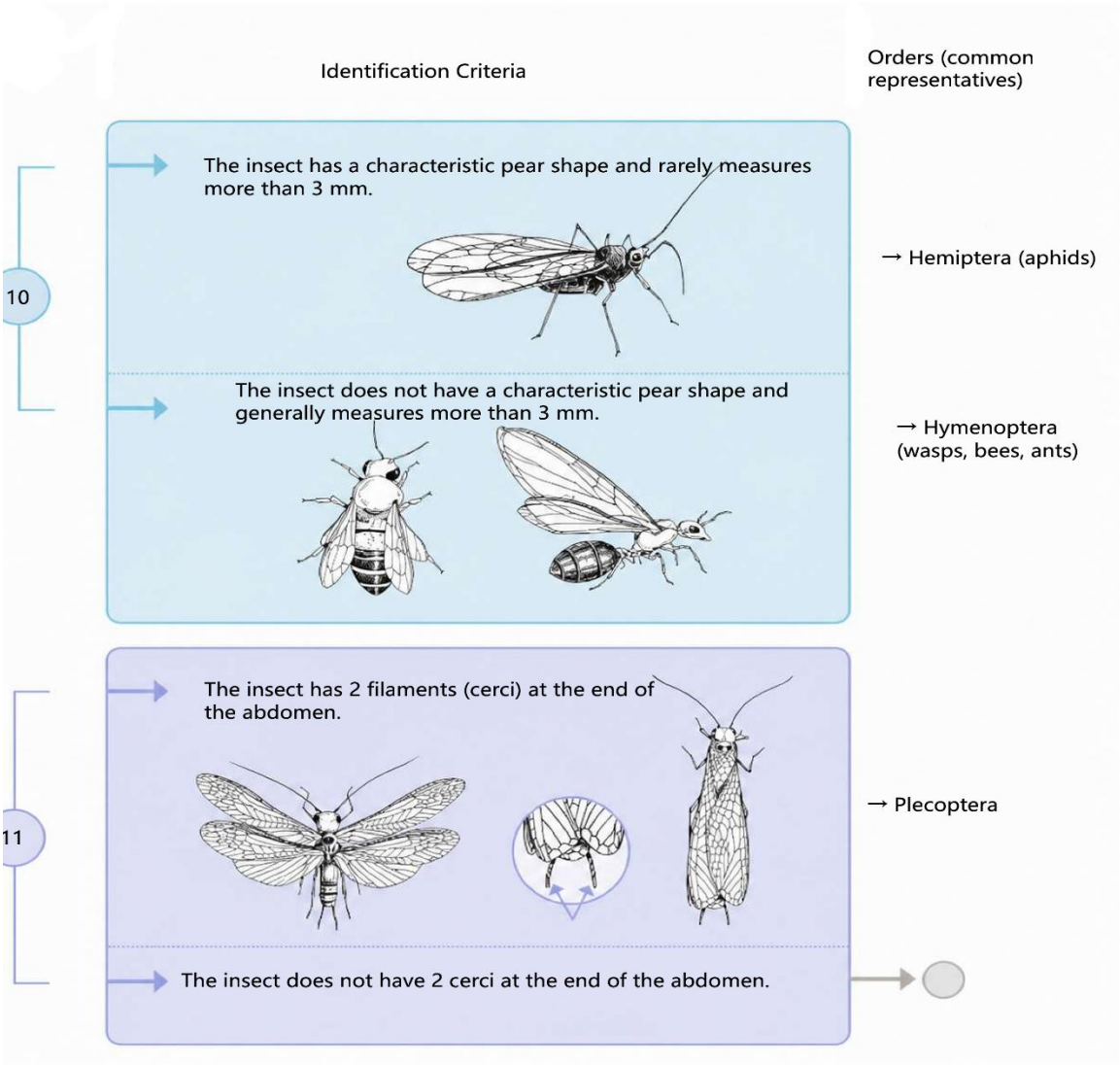

Figure 9 : Stages 10 and 11 include insects that look like pears and those that have two filaments at the end of their abdomen. (Hayat, 1983) ; (Pro Natura, 2021)

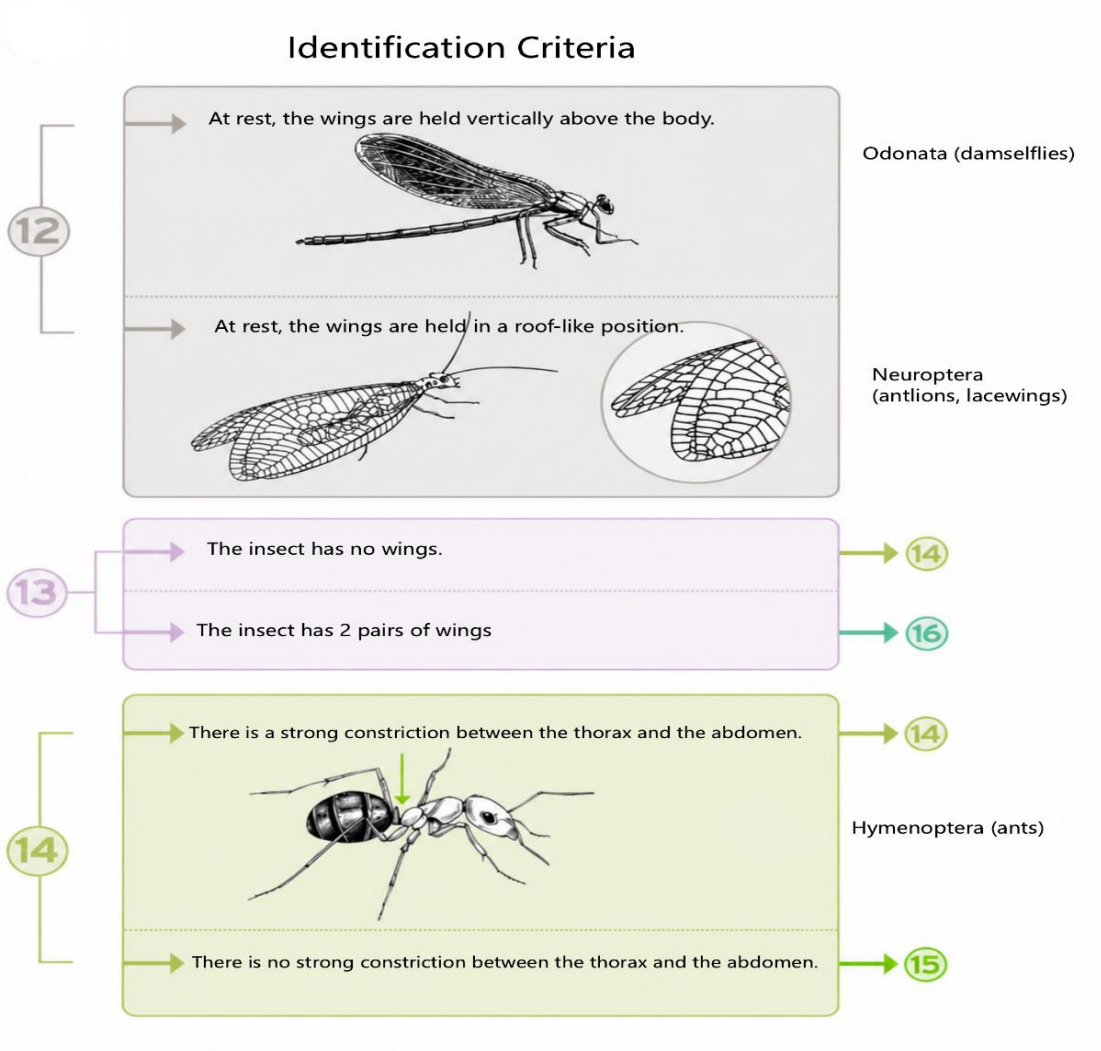

Figure 10 : The stages (12, 13 and 14) showed insects with wings that were like a roof, vertical wings, and those with a constrictor between the thorax and the abdomen. (LaSalle, 1987) ; (Pro Natura, 2021)

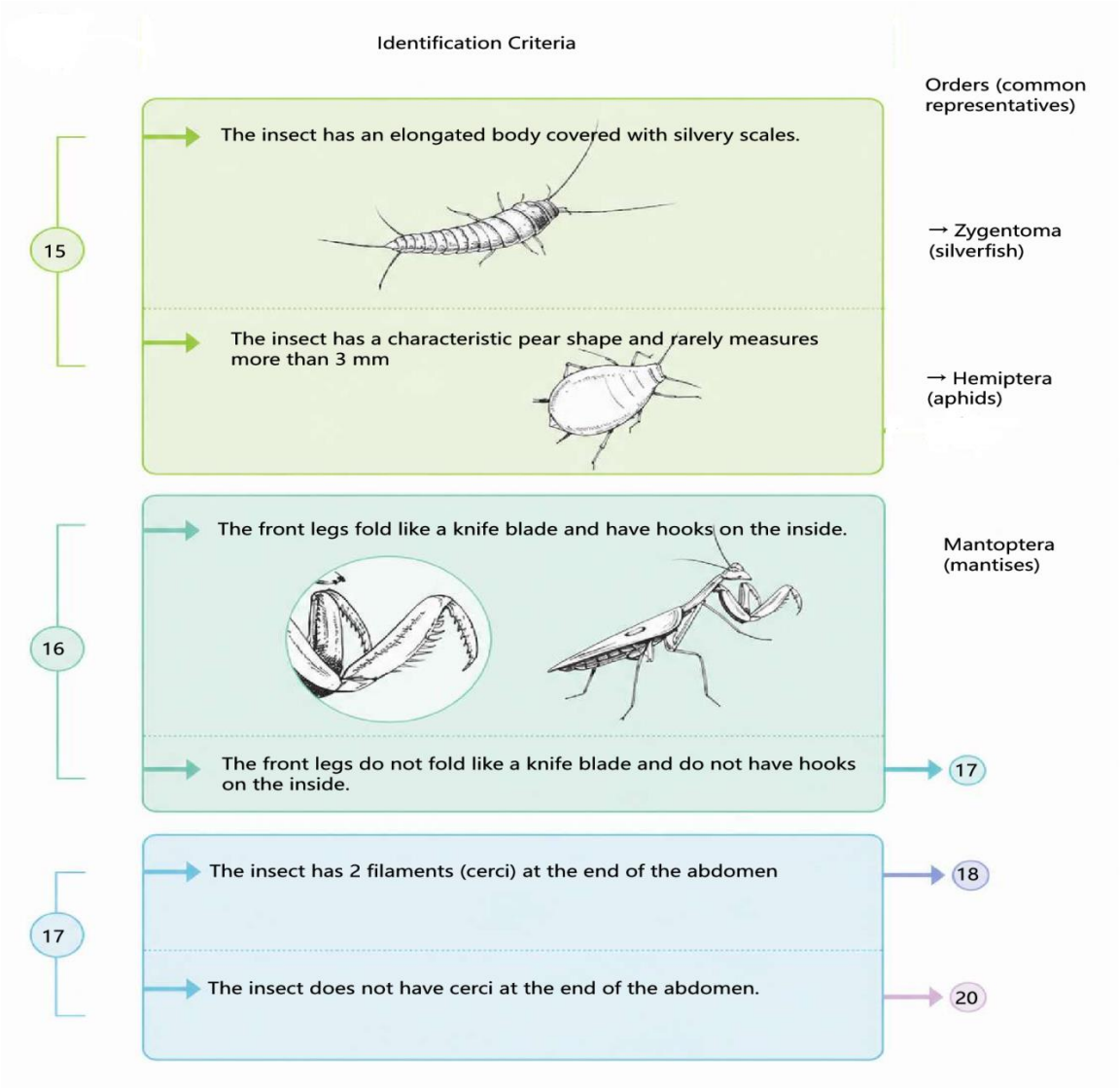

Figure 11 : Stages (15, 16 and 17) include insects with elongated bodies, insects with legs like a blade and insects without cerci. (Woolley, 1988) ; (Pro Natura, 2021)

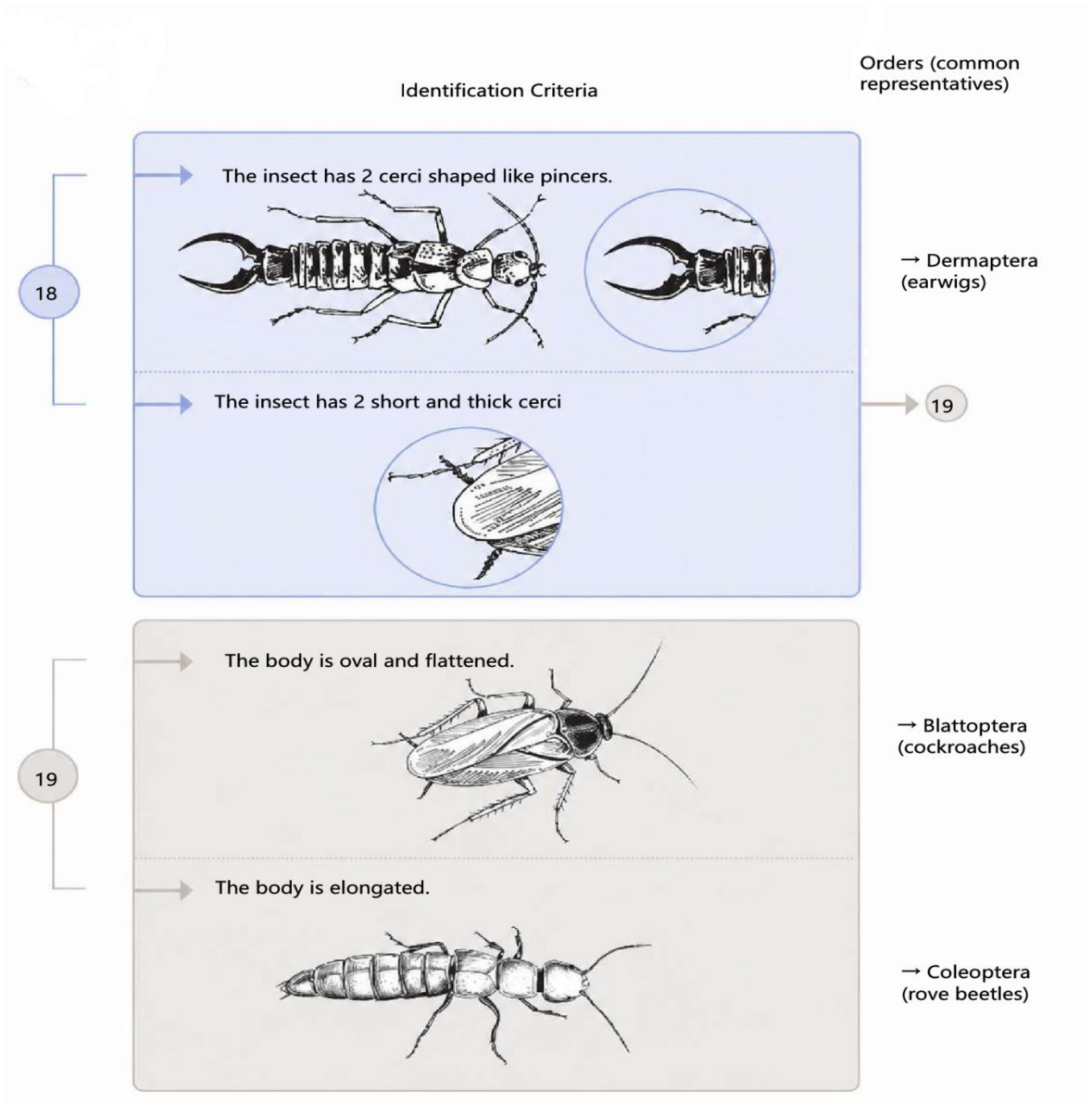

Figure 12 : Steps 18 and 19 involve both insects with pincer-shaped bodies and insects with a flattened oval body. (Mignon et al., 2016) ; (Pro Natura, 2021)

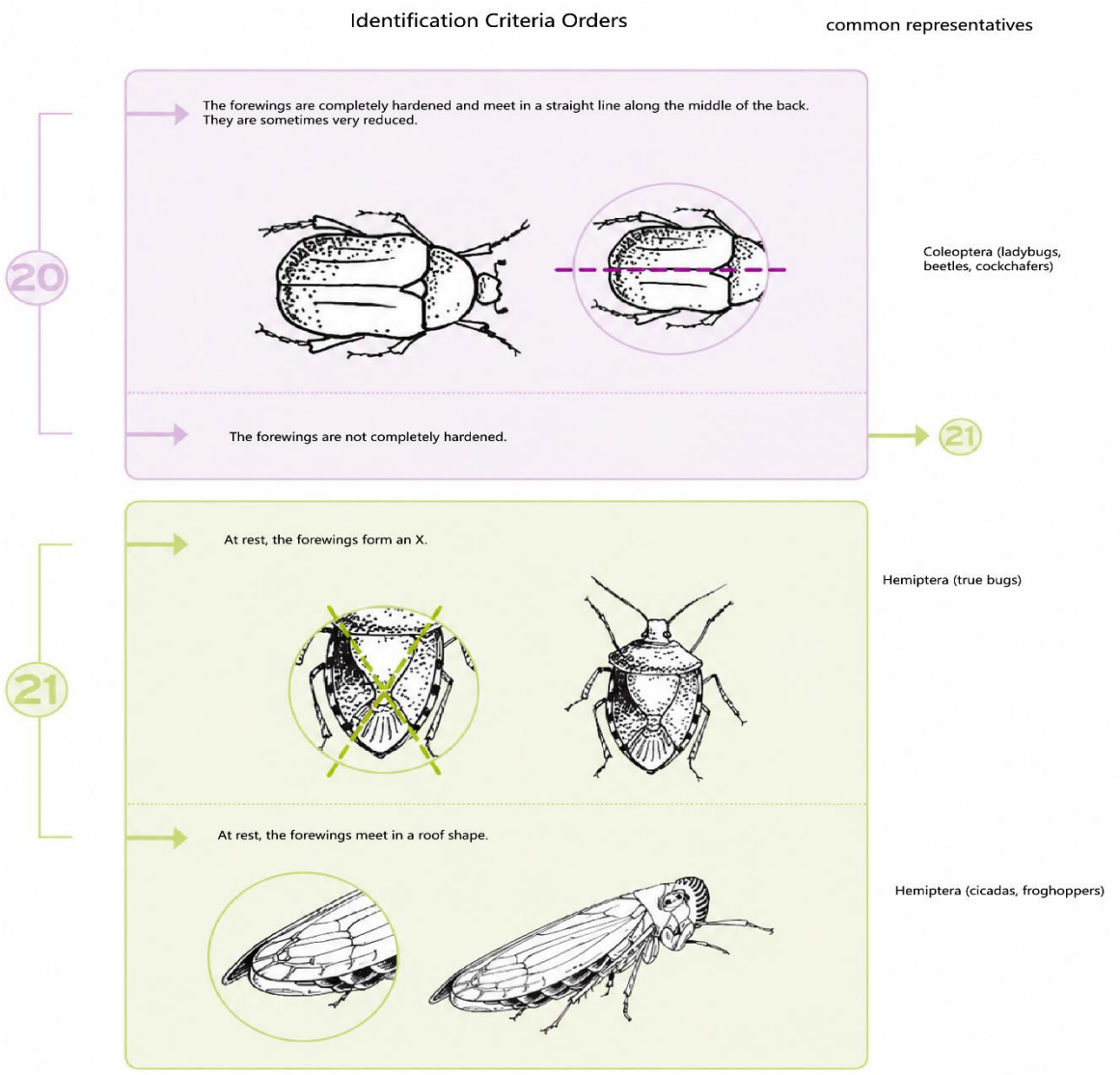

Figure 13 : Steps 20 and 21 include insects with hardened wings and insects with X-shaped wings. (Mignon et al., 2016) ; (Pro Natura, 2021)

IV.1.2. Nematode identification keys

In order to identify plant-parasitic nematodes, one must be able to recognize the most important diagnostic morphological structures. These structures are as follows

1. the general shape of the body,

2. the stylet,

3. the pharynx,

4. the cuticle and sensory organs,

5. and the reproductive system.

IV.1.2.1 Identification

In order to identify plant nematodes, you must be able to recognize the most important morphological structures (Figure 14). He is very important to practice the morphological characteristics under the microscope with a nematode from a genus of Hoplolaimidae (*Rotylenchus*, *Hoplolaimus*, *Scutellonema* ou *Helicotylenchus*).

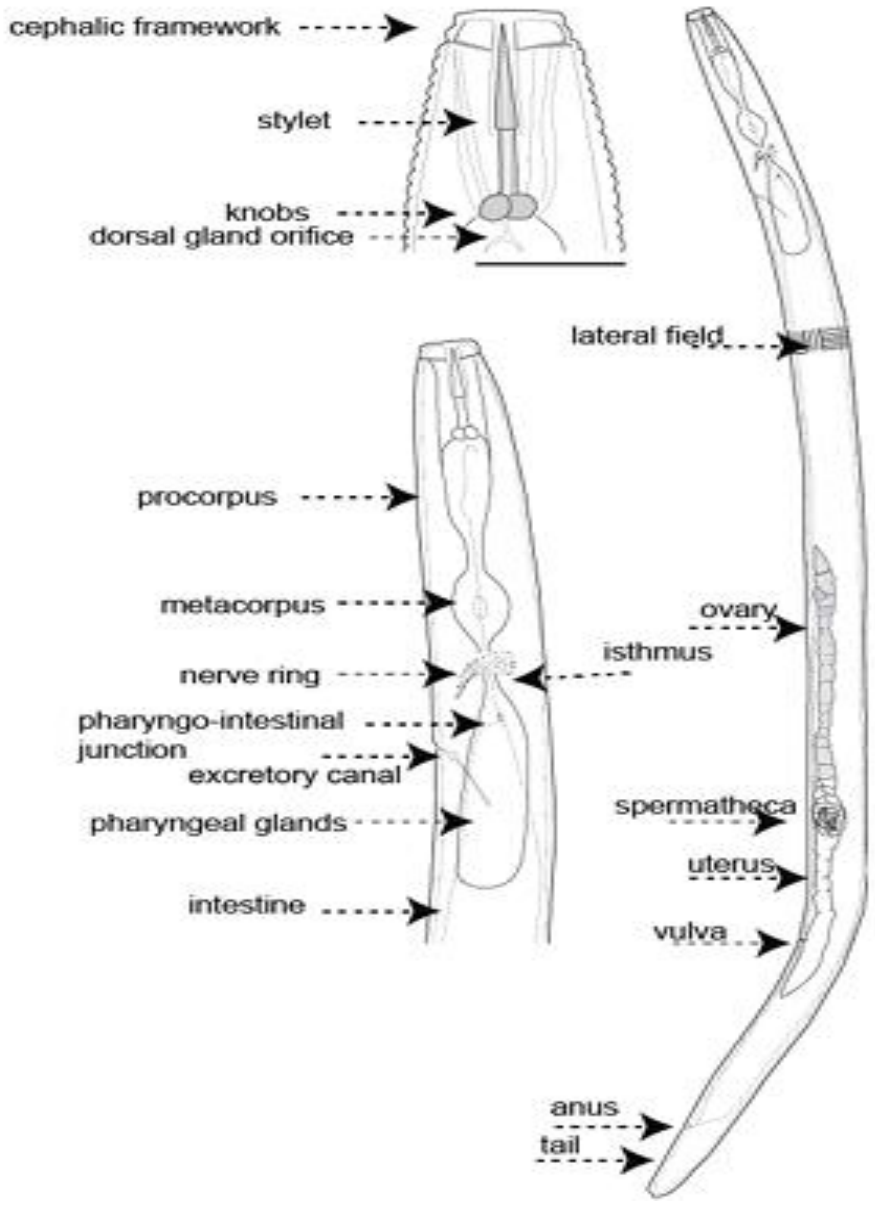

Figure 14 : The most important morphological structures annotated on a drawing of *Pratylenchus sp.* (Hunt & Palomares-Rius, 2012)

IV.1.2.2. The General Form of the Body

The body consists of an outer cylinder or tube (the wall of the body) and an inner cylinder (the digestive system) separated by a pseudo-celoma cavity filled with liquid under pressure. All root ectoparasites are vermiform throughout their life cycle. Other, more evolved plant-parasitic nematodes have a sedentary endoparasitic lifestyle and are capable of inducing specific cells or feeding structures in the root tissue (they are able to make the plant work for them) and they become obese (Figure 15). This type of life cycle is observed in gall

nematodes and cyst nematodes where the mature female becomes pyriform, globular or lemon-shaped. The body shape, or habitus, adopted by nematodes during relaxation (e.g. after fixation), varies from straight to spiral through a ventral curvature, which may be a useful diagnostic feature, particularly for stereomicroscopes.

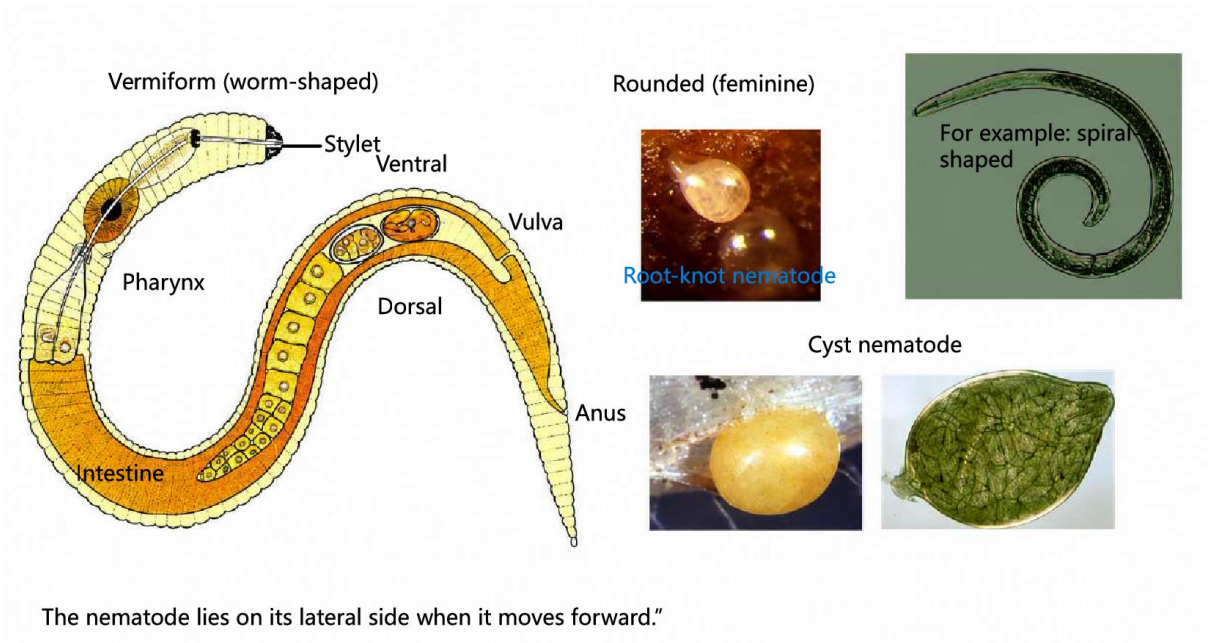

Figure 15 : The general shape of the body and the shape of the body after fixation (Hunt and Palomares-Rius, 2012) ; (NEMEDUSSA, 2024). www.nemedussa.ugent.be

IV.1.2.3. The Stylus

Plant-parasitic nematodes (PNPs) have a needle-like structure to puncture plant cells. The shape of this needle-like structure and its associated structures (e.g. the shape of its buttons) is crucial for identification. It can be a stylus, an odontostyle or an onchiostyle. Thanks to this needle-like structure, the nematode can extract food and secrete proteins and metabolites that help it parasitize the plant. Thanks to this needle-like structure, the nematode can extract food and secrete proteins and metabolites that help it parasitize the plant. In other words, most plant-parasitic nematodes use the stylet both as a straw to absorb plant juice and to manipulate plant behavior. Plant-parasitic nematodes are morphologically and functionally diverse and range from root-hair grazers to highly specialized plant-parasites, including ectoparasites and endoparasites with complex host associations. The three groups of plant-parasitic nematodes, Tylenchomorpha, Longidoridae and Trichodoridae, have evolved independently of each other. *Tylenchomorpha* have a stylet (Tylenchs &Aphelenchs, for example gall nematodes),

Longidoridae have an odontostyle (for example *Xiphinema*) and *Trichodoridae* (for example *Trichodorus*) have an onchiostyle (this is a modified tooth and not a hollow needle) to attack plants.

IV.1.2.4. The pharynx

The structure of the pharynx is related to the mode of feeding. The pharynx is sometimes also called the esophagus. In the Tylenchomorpha, the anterior part of the pharynx (or corpus) is subdivided into a procorpus, a metacorpus (medial muscular bulb), a non-muscular isthmus (which may be very short or absent), and three pharyngeal glands arranged either in a terminal bulb, either in one or more lobes overlapping the intestine. The arrangement of the pharyngeal glands is important for identification :

• overlap or not with the intestine

• and if there is dorsal, ventral or lateral overlap (for example, if the posterior part of the pharynx is mainly on the ventral side, this is called "ventral overlap").

In the Tylenchomorpha, the position of the exit from the dorsal gland is important

taxonomic (close to or far from the buttons of the stylet, or leading into the medial bulb).

In the Longidoridae, the pharynx is flask-shaped.

In the Trichodoridae, the pharynx consists of a narrow isthmus that gradually widens into a largely glandular basal bulb.

IV.1.2.5. The cuticle and sensory organs

For the cuticle or outer layer of the body, the number of lateral lines in the lateral area is particularly important for identification. Phasmids are sensory structures on the tail (one on each side of the tail). The phasmid can be larger, thus becoming a shield-like scutellum.

IV.1.2.6. Reproductive system

The female reproductive system is either didelphic with an opening to the outside via a medial-ventral vulva or monodelphic. Each genital branch consists of an ovary, an oviduct and a uterus and may have one or two sphincters (valves) and a spermatheca. The male reproductive system consists of two or one testicle, an gonotropic duct and a copulatory apparatus, which consists of spicules and gubernaculum (Figure 16).

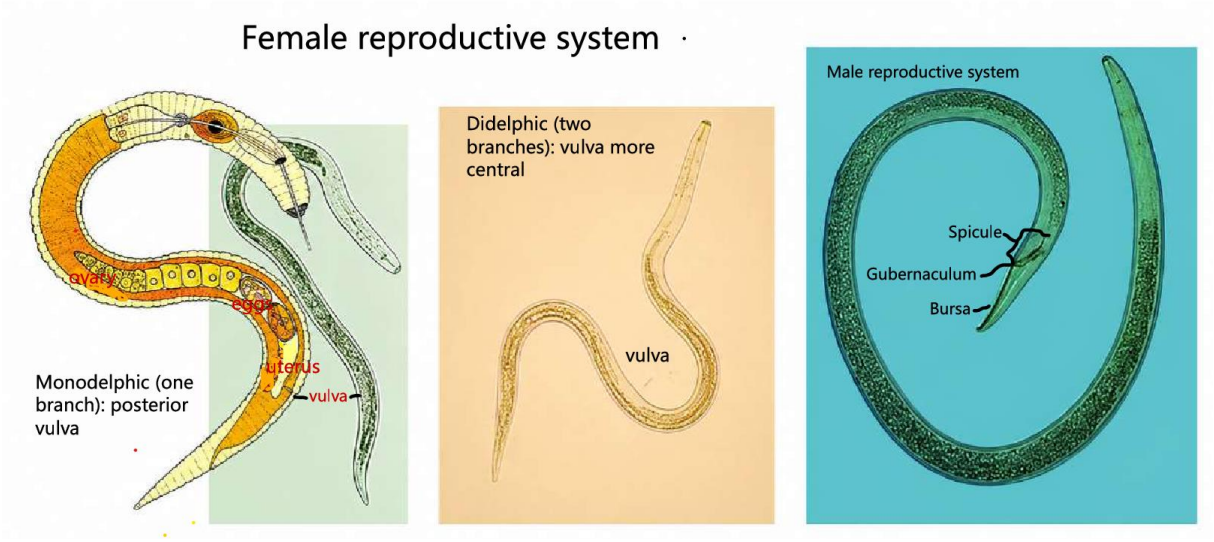

Figure 16 : The male and female reproductive systems (Hunt and Palomares-Rius, 2012) ; (NEMEDUSSA, 2024). www.nemedussa.ugent.be

IV.2. Traps and sampling methods

IV.2.1.Traps and sampling methods for insects

IV.2.1.1. Insect traps

IV. 2.1.1. 1. Description of the Barber pot method

This is the most commonly used type, simple to use ; this type of trap is a tool for studying medium and large arthropods. This type of trap mainly captures various walking arthropods, beetles, spiders and a large number of flying insects that come to the surface or fall there carried by the wind (Benkhelil, 1991). The material used is a container 15 cm in diameter and 18 cm high**.** In this case, it is metal cans that are placed on the land. Each trap pot is buried vertically, so that the opening coincides with the ground level, or at ground level. Earth is compacted around the opening to avoid the barrier effect that small arthropod species may encounter (Benkhelil, 1992). The Barber pots are filled with water at a third of their height. It is added to the detergent, which acts as a wetting agent that prevents trapped invertebrates from escaping. The trappers are placed according to the transect method. It is a line marked by a string along which about ten traps are installed at 5-metre intervals (Benkhelil, 1992).

IV. 2.1.1.2. Description of the mowing net method

The mowing net is a material used to capture beetles, dragonflies, orthopterans and insects standing on the ground (Benkhelil, 1992). The pocket of the reaping net must be made using a

large, solid fabric with tight meshes. The circle has a diameter of 30 cm formed by round wire from 0.3 cm to 0.4 cm in cross-section diameter. The depth of the bag varies between 40 and 50 cm. Its bottom is flat or slightly rounded so that its contents can be quickly accessible and examined after a few mowing strokes. The net handle is approximately 70 cm to 160 cm long. The net must always be handled by the same person and in the same way (Lamotte et al., 1969). This method consists of animating the net by back and forth movements, close to the horizontal, while maintaining the plane perpendicular to the ground. The maneuvers must be very fast and violent so that the insects surprised by the shock, fall into the pocket. In the present study, we carried out mowing for insect capture. Only one monthly exit is carried out between the 10th and 12th of each month. Each time, 5 times 10 sweeps are performed. It should be remembered that the quantity of insects caught after 10 rounds of harvesting is equivalent to a living population on a area of 1 square meter.

IV.2.1.1. 3. Description of the quadrat method applied to orthopteran fauna

The implementation of the quadrat consists in counting the individuals of each species of orthopteron present on a determined surface. Indeed, it consists in delimiting with a 12 m long string, squares or quadrats of 3 m on each side, for an area of 9 m2 (BRAHMI, 2005). Samples are taken once a month at each study station. During each trip, the date and exact location of the sampling are recorded (Figure 17).

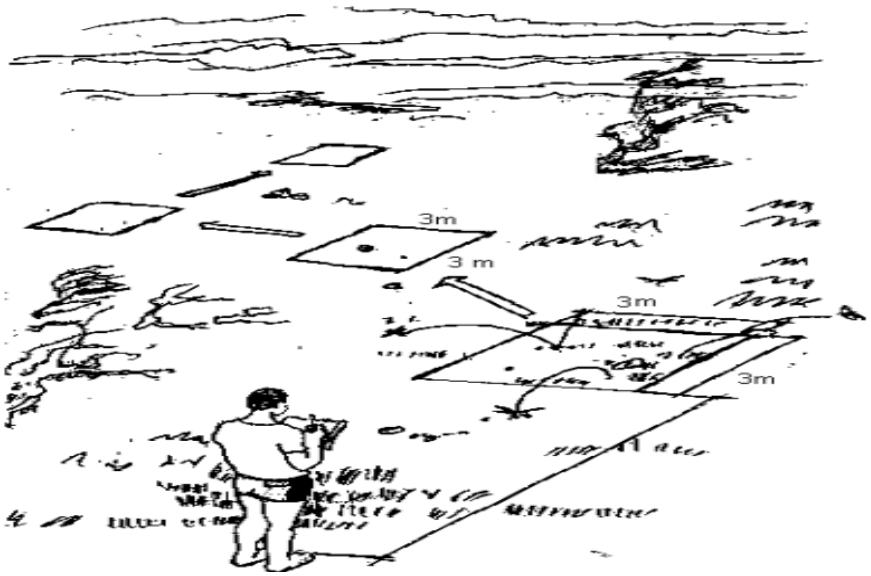

Figure 17: Sampling method using the LECOQ and MESTRE (1988) rough quadrat method. (Amended by AGGA)

IV. 2.1.1.4. Direct capture method (hand capture)

The method of direct insect capture at study stations is a complementary technique for other sampling methods to acquire interesting data on species biodiversity. The species caught are kept in a petri dish bearing the date and the name of the station.

IV.2.1.1.5. Sampling methods (insectes)

Method valid for all pests. It consists of examining a well-defined number of vegetative organs per crop unit or plot. The organs to be observed can be checked directly on the vine or taken for binocular examination or analysis by dipping.

A. Sampling. To obtain a satisfactory precision, the vines must be well distributed and chosen at random. In the cup plots, several ways of proceeding can be conceived (Figure 18 (diagrams below)).

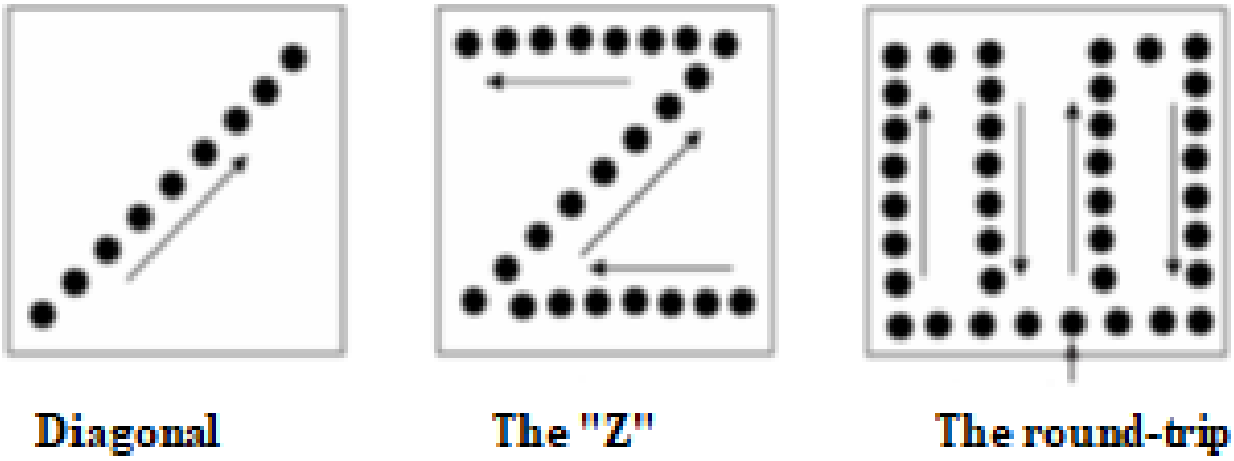

Figure 18 : Classic sampling (the diagonal, the "Z" and the round trip. (Southwood and Henderson , 2000) ; Agridea, 2011. www.agridea.ch

For trained shapes (wire), one organ is observed in at least 2 rows sufficiently spaced out, alternately on either side of each row (Figure 19 (diagram below)).

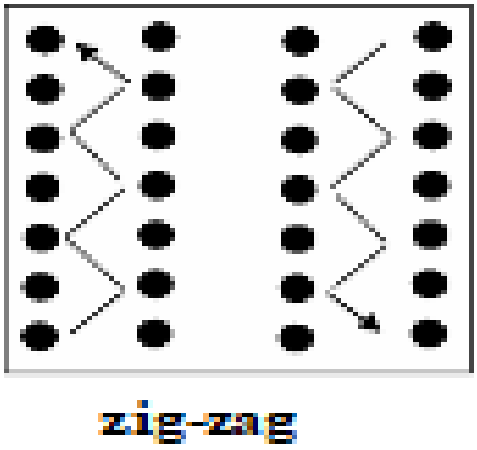

Figure 19 : Classic sampling (zig-zag). (Southwood and Henderson ,2000) ; Agridea, 2011 www.agridea.ch

IV.2.1.1.6. Sequential sampling (progressive)

Allows in most cases to reduce the sample and accelerate decision-making. Monitoring is carried out in sets of 10 organs, the number of organs occupied being cumulative. The value obtained after each series is compared with that of the reference table. This method is applicable to the control of red and yellow mites. It is not recommended for cluster worms, as it is not sufficiently reliable.

A. Sampling. The first group of 10 organs is taken from the 1st third of the plot, the second group comes from the 2nd third, and the 3rd group from the last third (Figure20 (diagram opposite)). Thus, the minimum sample is 30 organs. If necessary, sampling will be continued in the unvisited areas of the plot.

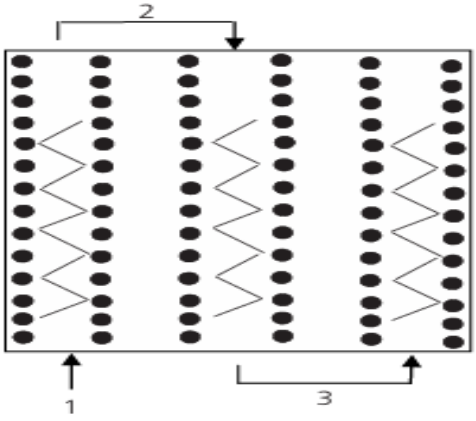

Figure 20 : Sampling for sequential sampling (Southwood and Henderson, 2000) ; Agridea, 2011 www.agridea.ch

B. Procedure. Choose the appropriate tolerance threshold, for example 30% occupied leaves (depending on the pest and stage).

1. Determine in each series of 10 sheets the number of sheets occupied and accumulate.

2. Example: 10 sheets, including 2 occupied sheets, then another 10 sheets, including 6 occupied sheets.

10 + 10 leaves = 20 leaves, of which 2 + 6 = 8 occupied leaves.

3. After each series, compare the obtained value with that of the table.

Example: 2 occupied leaves > 3, so the sampling continues.

8 sheets occupied > 5, table value, column T, the indication is to process and the check is complete.

4. If the value is lower than that in column NT, the indication is not to process. If, after 100 sheets, the value remains between those of the two columns of the table, we decide according to the value of the nearest table 3.

Table 3 : Reference for decision-making by sequential sampling of red and yellow mites (Marshall et al., 1994) ; Agrida, 2011 www.agridea.ch

Number of sheets checked (cumulative series of 10)	Tolerance threshold chosen in % of occupied leaves (lower limit - upper limit = threshold)													
	20% (5-20)		30% (10-30)		40% (20-40)		50% (30-50)		60% (40-60)		70% (50-70)		80% (60-80)	
	Number of sheets occupied for applying treatment (T) or deciding not to treat (NT)													
	NT ≤	T ≥	NT ≤	T ≥	NT ≤	T ≥	NT ≤	T ≥	NT ≤	T ≥	NT ≤	T ≥	NT ≤	T ≥
10	-	3	-	3	-	6	-	7	-	8	-	9	-	10
20	-	4	-	5	-	8	-	10	-	13	-	15	-	16
30	1	5	3	7	5	11	7	15	10	18	14	21	18	23
40	2	6	5	9	7	14	11	19	15	22	20	27	25	31
50	3	7	7	11	11	17	15	23	20	28	26	33	32	38
60	4	8	9	13	14	20	19	27	25	33	32	39	39	45
70	5	10	10	15	17	23	23	31	330	38	38	45	46	52
80	6	11	12	17	19	26	27	35	35	43	44	51	54	59
90	8	12	14	18	22	29	31	39	40	48	50	57	61	66
100	9	13	16	20	24	31	34	42	45	52	56	63	68	73

www.agridea.ch

IV.2.2. Traps and sampling methods for nematodes

IV.2.2.1. Collection of samples (sampling methodology)

The methodology used is that of the transect, which consists of sampling at a depth of 15 centimeters on both diagonals, both medians, and the center; multiple sampling was carried out on the diagonals, medians, and the center (Figure 21).

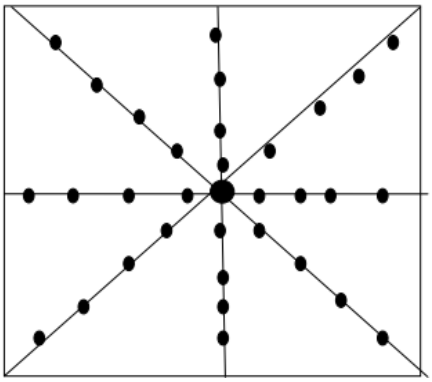

Figure 21: Sampling points under samples (Toure, 2012)

- The process is as follows : Multiple samples are taken every 2 or 3 meters on a diagonal. The sub-samples are mixed, homogenized and a representative sample is taken. We proceed in the same way for the other diagonal and the two medians. Then a sample is taken from the center, for a total of 05 samples. Subsequently, these 05 samples are homogenized and a representative sample of the plot is obtained, labeled, and will be subjected to nematological analyses.

- The collection of samples : A sample consists of a volume or quantity of soil, about 1 liter (or 1.5kg), taken near a plant and plant material (whole plant, underground part, removed from the same plant). This sample, as soon as it is taken, is collected in a plastic bag and then tightly sealed with a rubber strap to prevent drying out. When the plant part of the sample consists solely of roots, it may be placed in the same bag as the soil ; sorting will be done at the time of analysis. On the other hand, fragments of stems, leaves, and seeds must be collected in a separate bag.

- Faunal survey samples (Nematofauna): in this case, in a limited region, the aim is to determine which nematodes are associated with a given crop (for example, vegetable crops, lilies, legumes, vines, arboriculture, etc.). Little attention is then paid to the symptomatology. The main thing is to collect a large number of samples spread over the entire surveyed area.

This allows for the possibility of mapping the distribution of plant-parasitic nematodes associated with the crop. For large areas, good practice is to follow a road with a vehicle and to stop every 3, 5 or 10 km to carry out a sampling.

- Plant pathology survey samples: initially, the investigator's attention is drawn to areas of bad vegetation (spots) in a field that should normally constitute a uniform substrate. The existence of these patches of vegetation implies a pathological process whose causal agent must be investigated by analyses. In this case, the sampling is carried out outside the spots, at the edge of the spots and inside the tasks. After analysis, a possible correlation between the presence of deficiency symptoms and the presence of plant parasitic nematodes in greater or lesser numbers may suggest the involvement or exclusion of nematodes from the observed pathological process. Unlike the previous case, all samples may be collected on a small area.

- Control samples may be taken, either for practical or experimental purposes, to inject chemical substances with nematicidal effect into the soil. It is then necessary to control the effectiveness of these products by comparing the numbers of nematodes contained in samples from untreated areas and samples from treated areas.

Finally, it is necessary to choose the best time of collection. In particular, it is generally necessary to avoid taking samples from bare soil, which does not carry a crop, during the inter-campaign period. Indeed, in this case some nematodes migrate deeply and can even take refuge on the roots of perennial plants (trees) that grow underground. In other cases, the inter-campaign may be characterized by an accentuated drying of the soil to which certain nematodes respond with a state of quiescence, anhydrobiosis, which complicates the analysis of the samples. For these reasons, it is preferable to harvest crops during the growing season and preferably at a late stage of the cycle, in order to collect as many animals as possible. Indeed, since the generation time of plant-parasitic nematodes is about one month and the crop cycle is about 3 to 4 months, the population will reach its maximum around 2 or 3 months.

- Sampling protocol

Soil sampling:

15 to 30 samples (15-20 for detection/20-30 for diagnosis) at a depth of 0-30 cm.

- at random, in a zig-zag pattern over the entire plot if no dieback zone is present observed or on the outskirts of the area with symptoms.
- mix the samples together and take 500 g of this mixture = sample to be sent

Plant harvesting: About twenty plants are the sample to be sent; carefully remove the entire plant while preserving the soil adhered to the roots (Viglierchio and Yamashita,1983).

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