# Identification and Screening

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# Identification and Screening of Citrus Vein Phloem Degeneration (CVPD) on Brastagi Citrus Variety Brastepu (*Citrus nobilis* Brastepu) in North Sumatra Indonesia

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**Abstract**

Identification and screening of Citrus Vein Phloem Degeneration (CVPD) on Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu) in North Sumatra Indonesia is explained. The aim of the study is to explore the technique to obtain healthy Brastepu citrus to be used as a source of explants for in vitro propagation as a step in the preservation of threatened local citrus. The studies were conducted through collection of survived Brastepu citrus followed by screening the CVPD in the plants. Various procedures in the field and in the laboratory have been conducted to screen infected CVPD in Brastepu citrus. Visual examination from leaf, fruit, and seed appearance becomes preliminary information for CVPD infection in the citrus plant. Iodine test was used to support results obtained by visual observation. Histochemical test and the PCR analysis have confirmed that 12 samples of Brastepu citrus are free from CVPD and eight citrus are infected by CVPD. Adequate informations have been obtained to confirm healthy citrus tree which are then to be used as sources of explants for *in vitro* propagation in the preservation of *Citrus nobilis* Brastepu.

**Keywords:** Brastagi citrus, *Citrus nobilis* Brastepu, threatened citrus, screening CVPD, iodine test, histochemistry, PCR

## 1. Introduction

Brastagi citrus to be known as "Jeruk Brastagi" is very popular local sweet orange many years ago and become one of the well known commodities from Brastagi, North Sumatra, Indonesia. There are some local citrus that are commonly planted and developed in Brastagi namely variety Brastepu (*Citrus nobilis* Brastepu), Boci (*Citrus nobilis* Lour), and Rimokeling (*Citrus nobilis*) (Nurwahyuni & Sinaga, 2014). *Citrus nobilis* Brastepu which was called as Brastepu citrus has superior potential genetic compared with honey citrus and other local citrus, where the fruit is large in size, has sweet taste, and a raped peel is yellow to reddish color. Moreover, Brastagi citrus becomes multifunction plants as sources of fruit and the leaves and peels contains bioactive that are commonly used for Karo traditional medicine to cure some diseases (Simatupang, 2009). However, Brastagi citrus nowadays are grown in the field as unintended plants because they are not planted properly with some reasons, the production quantity and their sensitivity to some diseases such as CVPD. It has been informed by citrus farmer that the CVPD has destroied Brastagi citrus and nowadays it has been replaced by imported citrus. The eruption of Mount Sinabung on 2013-2014, where Brastagi as the impact areas, make the existence of local citrus be decreased in number. If Brastepu citrus is not conserved properly, it is predicted that this variety will disappear in the short time. Therefore, it is need to screen healthy survived trees to be used as sources of explants for in vitro propagation in the conservation of Brastepu citrus. The use of plants pathogen free is believed to be an efficient strategy to control citrus disease, and this strategy is chosen to obtain healthy citrus trees with free from CVPD which is then to be used as explants sources in the propagation of Brastepu citrus.

Some serious diseases that can destroy citrus has been identified such as Citrus Vein Phloem Degeneration

(CVPD), Citrus Sudden Death (CSD), Citrus Variegated Chlorosis (CVC), and Citrus Bacterial Canker (CBC), but the CVPD is the most devastating citrus diseases because there is no cure for the infection trees has been found (Doddapaneni et al., 2008; Vojnov et al., 2010; Halbert & Manjunath, 2004). Infection of CVPD or Huanglongbing (HLB), known as greening diseases (yellow shoot), is a very dangerous disease caused by the infection of bacteria *Candidatus Liberibacter africanus* and *Candidatus* L. *asiaticus*, an it has infected various type of citrus in many countries including Indonesia. The main target of CVPD infection is phloem tissue with the symptom on small-size and narrow yellow leaves, yellowing on some of the new shoot tip in the green canopy, greening on the leaves, chlorosis, leaf senescence, nutritional deficiency, low production of fruit, and shortening the lifespan of infected trees. Infection of CVPD can be seen from vegetative descriptions mainly in blotchy mottle leaves, small size fruits with the fruit do not color up and remains green when raper, dry mesocarp with bitter, salty or sour taste, and accompany with abnormal and sterile seed. The CVPD was responsible for the decline of citrus tree, lost production of citrus and destroy citrus plants in a short time such in Asia, South-East Asia, south and east Africa, Arabian Peninsula and America (Phahladira et al., 2012; Halbert & Manjunath, 2004). The CVPD was discovered in many countries such as United States, Mexico, South America, Africa, the Middle East, Reunion and Mauritius islands (Ammar et al., 2013; Hall et al., 2012). It is difficult to control the CVPD and infected trees results in low quality and quantity fruit productions (Bassanezi et al., 2006, Bassanezi et al., 2009; do Carmo et al., 2005; Kim & Wang, 2009). In Asia, infection of CVPD has been reported in China, Taiwan, Philippine, and Indonesia, and has destroyed sweet orange (Titrawidjaja, 1984; Bove, 2006).

Many studies have been conducted to identify the infection of CVPD in citrus. Screening methods to identify CVPD infection can be conducted by iodine test eventhough the test is not specific but it can be used to predict plant infection. The polymerase chain reaction (PCR) method is known as a specific method for determination of CVPD. It can be used to identify the presence of *Liberibacter* such as *L. Asiaticus* and *L. Africanus* (Kim & Wang, 2009). Grafting and vector transmission tests and PCR have been used to investigate CVPD symptoms (Ahmad et al., 2011). Identification of infected CVPD with intra-species specific molecular mechanisms associated with Las-induced responses in lemon plants based on the protein accumulation and the concentrations of cationic elements by using MS and ICPS analyses have also been reported (Nwugo et al., 2013). The PCR method has been used to identify CVPD infection in mandarin (*Citrus reticulata*) fruit based on physical and biochemical characteristic (Shokrollah et al., 2011). The PCR method has been claimed to be a rapid detection and identification of CVPD infection in citrus (Fujikawa et al., 2013; Kawabe et al., 2006). The Random Amplified Polymorphic DNA (RAPD) method has also been used to identify polymorphic DNA fragments of an infected citrus by the bacteria (Hocquellet et al., 1999). Semi quantitative RT-PCR method for expression pattern of Nucleotide Binding Site Leucien-Rich Repeats genes has been introduced for CVPD determination (Zamharir et al., 2014). Another method by using 2-DE and mass spectrometry analyses, as well as ICP spectroscopy analysis were employed to elucidate the global protein expression profiles and nutrient concentrations in leaves of Las-infected grapefruit plants at pre-symptomatic or symptomatic stages for CVPD (Nwugo et al., 2013).

The use of antibiotics has been claimed to be promising as potential control strategy for citrus CVPD (Zhang et al., 2014), but until now the removal and destruction of infected trees, and the use of plants resistant are still known to be efficient strategies to stop the spread of citrus disease (Weinert et al., 2004). The aim of the research is to explain a rapid screening technique to determine the presence of infected CVPD in *Citrus nobilis* Brastepu, a threat species of Brastagi citrus in North Sumatra Indonesia, in order to obtain healthy plants to be used as sources of explants for *in vitro* propagation of Brastepu citrus as a step in the preservation of threatened local citrus.

## 2. Methods

Research methods are consisted of collection of Brastagi citrus variety *Citrus nobilis* Brastepu, identification and screening of CVPD. Collection of Brastagi citrus was conducted to obtain survived local citrus in the villages at Brastagi, Kabupaten Karo North Sumatra, Indonesia. Identification of CVPD was conducted in the field by using visual characterization based on plants performance on their leaves, fruits and seeds, followed the procedures explained earlier (Nurwahyuni & Sinaga, 2014). All laboratory experiments are conducted in Biology Laboratory FMIPA USU, Medan, and Agriculture Biotechnology Laboratory, Gadjah Mada University Yogyakarta. Screening of CVPD on *Citrus nobilis* Brastepu was conducted from its leaves by using iodine test with KI solution of iodine kit. Molecular analysis with histochemical test was performed for molecular tissue by using a microscope. Extraction of the DNA, PCR, and electrophoresis are conducted followed the procedures explained in the references (Fontarnau & Hernandez-Yago, 1982; Weising et al., 2005; Amani et al., 2011). Confirmation test for infection of CVPD is conducted by using PCR. The details of the procedures are explained in previous report (Nurwahyuni & Sinaga, 2014).

## 3. Result and Discussion

### *3.1. Characterization of Citrus nobilis Brastepu*

Citrus Brastepu (*Citrus nobilis* Brastepu) is a local variety of sweet orange in Brastagi North Sumatra which is known as Brastagi citrus. The characteristic of healthy Brastepu citrus is presented in Figure 1. Mature Brastepu citrus can reach up to 3-4 metres (Figure 1a) and a healthy plant is productive up to 25 years old. The discription of citrus flower is five petals with white color and yellow anther (Figure 1b). Brastepu citrus is a seasoning fruit that only produce fruit for big harvest once a year, and the typical of green young fruit is presented in Figure 1c. The characteristic of citrus fruit is large in size, about 4.1-5.3 cm in diameter, and the taste is sweet. The color of fruit skin when young is green and the peel becomes orange to reddish color when raped (Figure 1d). The Brastepu fruit is with flat basal, the thick of the skin is 0.30-0.50 mm, where in flavedo 0.25-0.40 mm. The skin of Brastepu fruit is easily peeled with orange lith (fruit segment), and contains less seed about 4-8 seeds each fruit (Figure 1e). Brastepu citrus have single leaf, the avarage of which is 7 cm and 2.5 cm in length and wide, repectively. The stipula is clearly seen in the leaf. Peels and the leaves of Brastepu citrus contains bioactive compounds that are commonly used for Karo traditional medicine to cure some diseases (Nurwahyuni et al., 2012; Simatupang, 2009). Despite its superiority in the quantity and quality of Brastepu fruits, such as large in size, sweet taste, and the content of medicinal bioactive compounds in the plants, nowdays Brastepu citrus was not planted properly and has tend to be replaced by honey citrus due to its low resistance to CVPD. It is predicted that Brastepu citrus to be demolished soon if an action has not been done to preserve the plant. With this reason, the study is carried out to obtain healthy Brastepu trees that are free from CVPD to be used as sources of explants for *in vitro* propagation in the preservation of *Citrus nobilis* Brastepu. It is believed that survived healthy Brastepu citrus has resistant ability to CVPD, and *in vitro* propagation becomes a good strategies to produce good quality seedling to preserve the potential genetics of local citrus from diminished and to stop Brastagi citrus from loss of plant diversity.

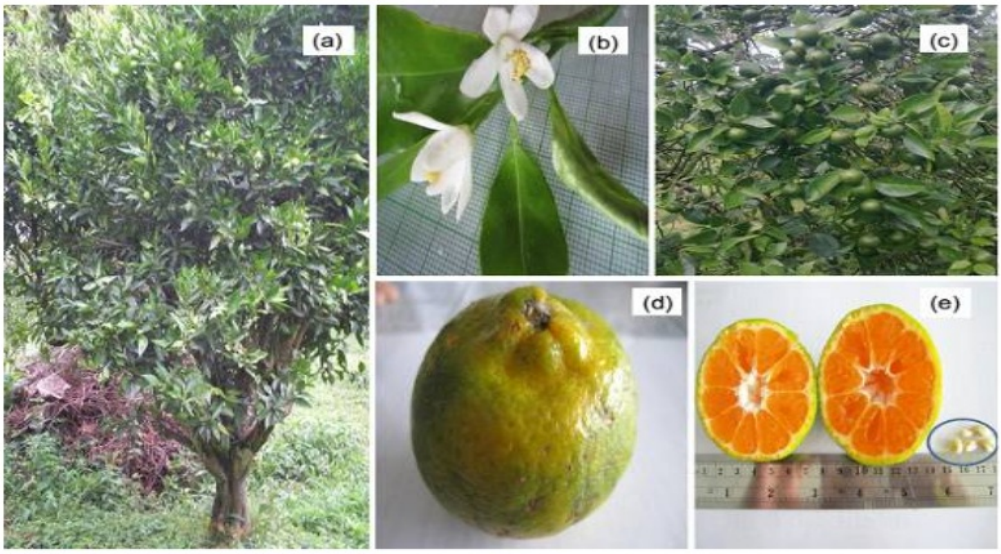


Figure 1. The description of Citrus Brastepu (*Citrus nobilis* Brastepu): (a) A mature healthy Brastepu citrus grown in Desa Bukit, (b) the flower, (c) Immature green fruit, and (d) Mature fruit, (e) Citrus anatomy and its seed

### *3.2 Visual Investigation to Screen CVPD on Citrus nobilis Brastepu*

The survey has been conducted to search survived *Citrus nobilis* Brastepu at Brastagi, Kabupaten Karo, North Sumatra, Indonesia. There were only 20 survived citrus found from six villages, successively at Desa Tongkoh (2 plants), Desa Bukit (10 plants), Desa Melas (2 plants), Desa Beganding (2 plants), Desa Surbakti (3 plants), and Desa Brastepu (3 plants). The condition of plants are varied from 5-30 years old, most of them are categorized as wild plants. Screening of CVPD on Brastepu citrus has been conducted by visual identification as it is known

that phatogen detection can be seen based on the typical of leaf tissue and fruits disorder. Identification of infected plant can be seen from its vegetative symptom with one or some of these descriptions of blotchy mottle, small and narrow leaves, greening fruits with small seed and undeveloped flavedo with bitter or salty tasted due to CVPD infection in the floem. The disease has affected on cell defense, transport, photosynthesis, carbohydrate metabolism, and hormone metabolism (Albrecht & Bowman, 2008; Martinelli et al., 2012). The characteristic of Brastepu citrus from different plants is summarized in Table 1. Most of the plants are look healthy with green wide leaves, good pruning, and big size fruits. Some of Brastepu citrus are suspected to be infected by the CVPD with symptoms of small leaves, narrow shape, mottle, and yellowish as shown in Figure 2. Infected plants show greening leaves with necrosis that make the texture of citrus leaf abnormal in isolated branches. Furthermore, the vascular bundel of the leaf is spongy type with yellow color in the whole leaf, transparence, and also containing of green spot in the vascular bundle (Figure 2a). The greening to yellow process is continued in the leaf starting from vascular bundle up to leaf margin, followed by chlorosis, blotchy, and almost all leaves developed small and narrow as a symptom of CVPD infection (Figure 2b).


Figure 2. The symptom of infected CVPD on Brastepu citrus: (a) Greening to yellow leaves with chlorosis in branches, (b) The development of small, narrow, and *blotchy leaves,* (c) Small size and fade greening fruits flavedo and dry, and (d) Formation of black, long and small seed with sterile or abort

During the collection time, most of Brastepu citrus plants did not produce fruits, only few of them produce fruits. A healthy plant produce many big fruits with normal development, and orange to reddish feel color with orange lith, fresh and sweet taste with normal seeds when fruit raped. Unhealthy citrus tree produce few fruits, most of them abort when they are young. The fruits tend to developed abnormal and have small size, and when they raped they remain green with dry locule with bitter and salty taste and they are asymmetric and lopsided with a bent fruit axis (Figure 2c). The formation of green color for raped fruit can be used as an identification of CVPD infection due to the problem in the synthesis of chlorophyl to become chromophyl (Roy & Goldschmidt, 2008). The development of the seed in an infected plants are also abnormal, the shape is long, black in color, aborted seeds and sterile (Figure 2d). The fruit is asymetric with dry and fade flavedo accompany with small size seed is the indication of infected CVPD diseases (Brlansky & Roger, 2007; Bove & Ayres, 2007). These results may be used to predict healthy and infected citrus that to be confirmed in the next examinations, those are the iodine test, histochemical anatomy, and PCR analysis.

Table 1. The description of Brastepu citrus from visual observation in the field for screening of CVPD infection from their leaves, fruits and seeds

| Plants Code | Location | The description for CVPD simpthom | | |
|---|---|---|---|---|
| | | Color of Leaf | Vascular and Venae of Leaf | Raped Fruits and The seed |
| 1 | Desa Tongkah | Green healthy | Normal | No fruit |
| 2 | Desa Tongkah | Green healthy | Normal | No fruit |
| 3 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 4 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 5 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 6 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 7 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 8 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 9 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 10 | Desa Bukit | Green healthy | Normal | Normal, orange, the seed normal |
| 11 | Desa Melas | Leaf defoliation, ±50% yellow | Brown | Small fruits, fade flavedo, the seed is black, sterile, abort |
| 12 | Desa Melas | Leaf defoliation, ±50% yellow | Brown | Small fruits, fade flavedo, the seed is black, sterile, abort |
| 13 | Desa Beganding | Green healthy | Normal | Normal, orange, the seed normal |
| 14 | Desa Beganding | Green healthy | Normal | Normal, orange, the seed normal |
| 15 | Desa Surbakti | Greening and yellow, *blotchy mottle* leaves | Normal, some are yellow | Normal, orange to green, the seed is steril |
| 16 | Desa Surbakti | Greening, and yellow, *blotchy mottle* leaves | Normal, some are yellow | Normal, orange to green, steril |
| 17 | Desa Surbakti | Green healthy | Green spot in the vascular bundle, leaf spongy type | Small size fruit, green in color, the seed is black, sterile, and abort |
| 18 | Desa Brastepu | Leaf defoliation, ±75% yellow | Brown | No fruit |
| 19 | Desa Brastepu | Leaf defoliation, *blotchy mottle* leaves, ±75% yellow | Brown | Small fruits, fade flavedo, the seed is black, sterile, abort |
| 20 | Desa Brastepu | Leaf defoliation, ±75% yellow | Brown | No fruit |

### *3.3 Iodine test for Screening CVPD Infection*

Screening of CPVD infection by using iodine test was conducted to support the visual investigation in the field as it is known that iodine is reacted with amylum in the leaf to produced dark blue color. This method is effective to diagnose the presence of amylum results from photosynthesis (Su, 2008). There are 5 leaves are randomly taken from every Brastepu citrus plants, and the test were conducted in pastic bag for each of them to observed and the color changing as shown in Figure 3, and the results are summarized in Table 2. It is seen from the test that there are 12 plants are negative to CVPD where the leaves samples are not given change in color with iodine because accumulation of amylum were not found in healthy tissues. Special attentions have been conducted for suspected unhealthy samples (No. 11, 12, 17, and 18), where the iodine give color change from brown to black color. Another four samples gave positive-negative results to CVPD. They are samples No. 15, 16, 19, and 20. The results in iodine test supported the results obtained by visual investigation. There are 12 Brastepu citrus plants free from CVPD and eigh samples suspicious to be infected by CVPD. Further infestigations have to be conducted to confirm these results by using histochemical anatomy test and the PCR analysis.

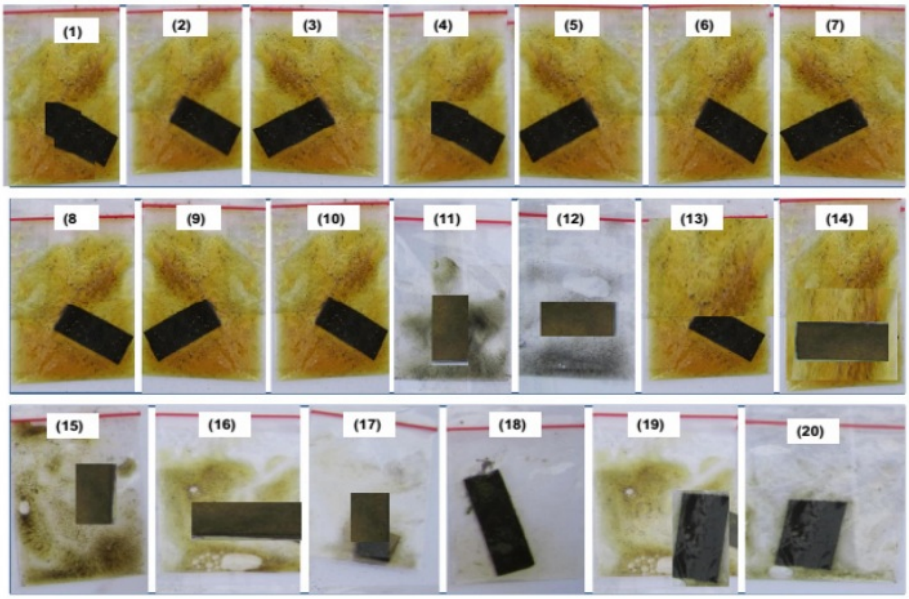


Figure 3. Screening of CVPD on 20 *Citrus nobilis* Brastepu by using iodine test. Brown color represent healthy leaves, and blue to black color are suspicious to be infected by CVPD

Table 2. Screening analysis of CVPD infection for sample of *Citrus nobilis* Brastepu that are obtained from various villages in Brastagi, Kabupaten Karo, North Sumatra, Indonesia

| No Citrus Plants | Villages Location | Analysis and Confirmation of CVPD infection | | |
|---|---|---|---|---|
| | | Iodine test* | Amylum – Anatomy Test** | Analysis PCR |
| 1 | Desa Tongkah | 5 Negative | Negative | Negative |
| 2 | Desa Tongkah | 5 Negative | Negative | Negative |
| 3 | Desa Bukit | 5 Negative | Negative | Negative |
| 4 | Desa Bukit | 5 Negative | Negative | Negative |
| 5 | Desa Bukit | 5 Negative | Negative | Negative |
| 6 | Desa Bukit | 5 Negative | Negative | Negative |
| 7 | Desa Bukit | 5 Negative | Negative | Negative |
| 8 | Desa Bukit | 5 Negative | Negative | Negative |
| 9 | Desa Bukit | 5 Negative | Negative | Negative |
| 10 | Desa Bukit | 5 Negative | Negative | Negative |
| 11 | Desa Melas | 5 Positif | Positive | Positive |
| 12 | Desa Melas | 5 Positif | Positive | Positive |
| 13 | Desa Beganding | 5 Negative | Negative | Negative |
| 14 | Desa Beganding | 5 Negative | Negative | Negative |
| 15 | Desa Simpang Empat | 5 Positive | Positive | Positive |
| 16 | Desa Simpang Empat | 5 Positive | Positive | Positive |
| 17 | Desa Simpang Empat | 2 Positive, 3 Negative | Positive | Positive |
| 18 | Desa Brastepu | 1 Positive, 4 Negative | Positive | Positive |
| 19 | Desa Brastepu | 1 Positive, 4 Negative | Positive | Positive |
| 20 | Desa Brastepu | 1 Positive, 4 Negative | Positive | Positive |

*Note*. * There are 5 leaves are randomly selected from one plant; ** There are 3 leaves are randomly selected from one plant.

*3.4 Histochemical Anatomy Test for CVPD Infection*

Followed by the results obtained by visual investigation and iodine test as stated above, another screening test for CVPD infection has been carried out through histochemical test for molecular anatomy investigation in a preparat that was chosen closed to petiol of the leaf by using microscope. The histochemical anatomy results for the tissue of a healthy and infected Brastepu citrus are presented in Figure 4. The tissues of healthy and infected tissues are clearly shown in the figure where the pholem cell in a healthy leaf is normal where the amylum is distributed well in the cell (Figure 4a). In unhealthy leaf tissue, the accumulations of amylum was found in certain areas of a pholem cell, that were shown with the observation of dark to black spots of amylum on the parenchym tissues (Figure 4b). The preparat of infected leaf consisted of empty room without amylum because the amylum could not distribute well due the miss orientation of the tissue as a results of infection of pholem cell. Histochemical anatomy test have been conducted randomly for three leaves in evey plants and the results are summarized in Table 2. The tissues of 12 Brasitepu citrus plants are healthy, and eight of them are suspected to be infected by the CVPD. Screening results from visual observations, iodine test, and histochemical anatomy test have given the information on the status of Brastepu citrus, and the results are all agree and consistent to show the indication of negative and positive infection of CVPD in Brastepu citrus. The PCR analysis is then to be used for confirmation of CVPD in the citrus trees.

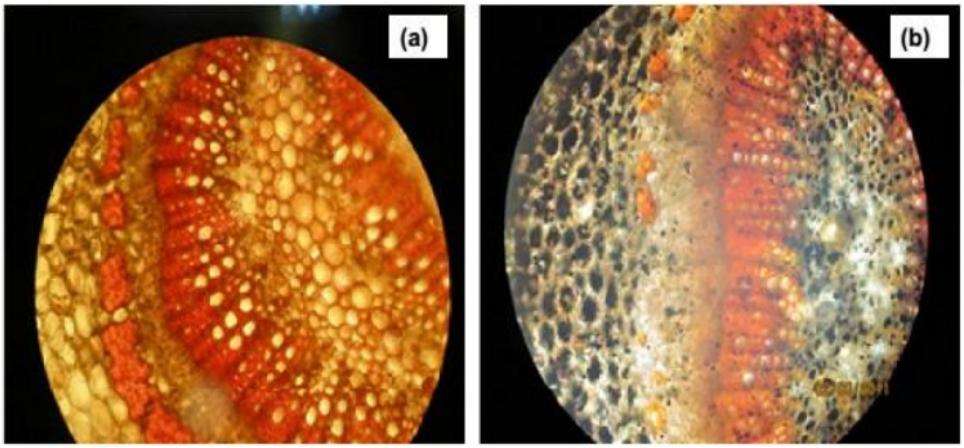


Figure 4. The anatomy of a petiol to show infection of CVPD in citrus leaves at 40 x amplification: (a) Healthy tissue, (b) Unhealthy tissue with accumulation of amylum

*3.5 Isolation of DNA for PCR Assays*

Isolation of the DNA from Brastepu citrus leaf has been carried out for PCR analysis. It is known that the presence of certain protein in the DNA can be used to confirm the CVPD infection (Akarapisan et al., 2008). Isolation of citrus DNA is conducted by extraction of lysis cell, separation and purification. The electrophoresis results for the DNA Brastepu citrus has proved that the DNA was isolated well, and the quantity of the Brastepu citrus DNA has been measured by using spectrophotometric method which are adequate for PCR analysis and confirmation. To confirm CVPD infection in Brastepu citrus, the DNA of Brastepu citrus is analysed followed the procedures explained with using CVPD protein-specific primers (Padmalatha & Prasad, 2006; Sahasrabudhe & Deodhar, 2010). The PCR results are shown in Figure 5 and the confirmation results are summarized in Table 2. The results have showed that there are 12 citrus plants have confirmed free from CVPD and eight citrus plants are infected by CVPD.

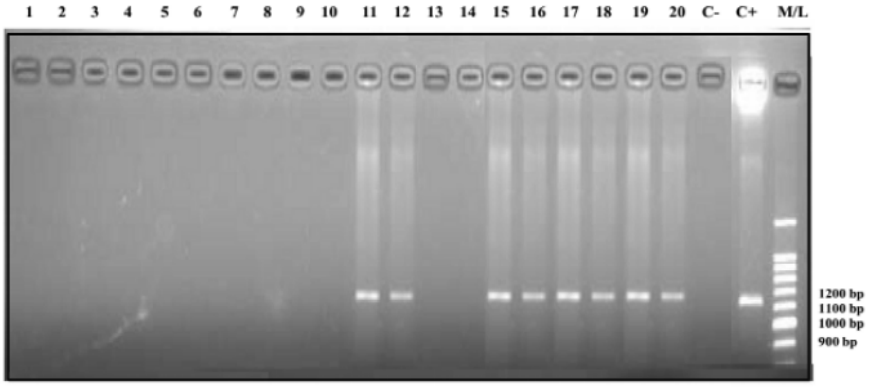


Figure 5. The PCR analysis of citrus DNA for confirmation of CVPD infection. Infected samples are shown in the figure

The PCR test has confirmed that Brastepu citrus number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, and 14 are free from CVPD, and another Brastepu citrus plants number 11, 12, 15, 16, 17, 18, 19, and 20 are positively infected by the CVPD. Therefore, healthy citrus plants has been selected to be used as the source of explant for *in vitro* propagation, those are the Brastepu trees grown in Desa Bukit Brastagi because the all plants are healthy with plenty of fruits. Potential genetics of healthy Brastepu citrus would be inherited to the comming Brastepu seedling that to be produced by *in vitro* propagation in the preservation of threated *Citrus nobilis* Brastepu in further study.

## 4. Conclusion

Screening method to investigate the presence of infected CVPD on Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu) in North Sumatra Indonesia has been conducted. Visual identification from the leaf, tissue, fruits, and seed of Brastepu citrus have given the informations that some plants are infected by CVPD. Screening program by iodine test for leaves tissues and anatomy of petiol were used to support the results from visual observation. The PCR analysis has confirmed that 12 Brastepu plants are free from CVPD and eight Brastepu plants are positive to be infected by the CVPD. The study is assigned to be a good strategy to select healthy citrus plant to be used as source of explant for *in vitro* propagation, that is a step in the preservation of threatened Brastagi citrus variety Brastepu.

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