# Detection of *Potyvirus* using RT-PCR and ACP-ELISA of *Dioscorea* species and *in vitro* shoot multiplication of the virus free plants

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## Abstract

Detection of *Potyvirus* using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Antigen Coated Plate-Enzyme Linked Immunoabsorbent Assay (ACP-ELISA) for *Dioscorea alata*, *D. hispida* and *D. esculenta* was conducted in order to establish *in vitro* culture of virus-free of these species. Plants were collected from Yogyakarta, Lampung, Pasuruan, Jakarta and Bogor. Total RNA of plants grown in a greenhouse was then isolated according to Simple Direct Tube (SDT) method. Total RNA from symptomatic leaf of Yard Long Bean (*Vigna unguiculata*) infected with Bean Common Mosaic Potyvirus (BCMV) was used as the positive control treatment. RT-PCR assay with degenerate primers MJ1(F) and MJ2(R) was used to identify the *Potyviruses* infecting *Dioscorea*. ACP-ELISA with antibodies specific to group *Potyvirus* was carried out to detect *Potyvirus* from leaves samples. The *Dioscorea* virus-free species was then cultured on modified MS medium. Shoot tips or internodes were used as explants. The results showed that using both RT-PCR and ACP-ELISA, all species tested were free from virus. The growth response of explants on MS medium was varied depending on the plant species and the concentration of BAP.

**Keywords**: *Dioscorea* spp., *Potyvirus*, RT-PCR, ACP-ELISA, *in vitro* shoot multiplication

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## Introduction

Family *Dioscoreaceae*, genus *Dioscorea* are an important alternative food crop in tropical and subtropical regions as well as useful for functional food. As a functional food, *Dioscorea* used for blood-pressure regulator to overcome hypertension (Liu *et al.*, 2009); used for reduction of the risk of obesity, diabetes and other related diseases (Aprianita *et al.*, 2009); used for source of antioxidant (Lubag *et al.*, 2008). West Africa is the most important cultivation zone, where *Dioscorea* (yam) is a major staple, producing about 93% of the world's edible yams. The crop is also of considerable importance in parts of eastern Africa, the Pacific area (including Japan), the Caribbean and tropical America for food, medicine and bioethanol (Arnau *et al.*, 2010).

*Dioscorea alata*, *D. esculenta* and *D. hispida* are three major species of edible *Dioscorea*. The place origin of *D. alata* is Asia and widely cultivated in India, South East Asia and the Pasific islands. It grows well in the lowland up to 800 m above sea level. *D. esculenta* is a native of Indo-China, and widespread to Madagascar and New Guinea. It is usually cultivated in the lowlands up to 700 m alt. Meanwhile, *D. hispida* is native to western India, and widespread in South East Asia. It grows in the lowlands up to 850 m alt (Sastrapradja *et al.*, 1981).

Many of *Dioscorea* species grow in several area in Indonesia (Somantri *et al.*, 2005), however, cultivation in large area has not been done extensively. One of the factors that cause these plants receive less attention is that the existence of other types of crop producing carbohydrates have better quality, growth faster, easier and higher productivity. Beside that major factor, tuber of *Dioscorea* species utilization still limited as a snack for Indonesian people (Susanto, 1983).

*Dioscorea* is clonally propagated by tubers. The sprouting tubers are used as propagules, planted just before the rainy seasons. The plant can be harvested after one year (Sastrapradja *et al.*, 1981). A single plant bear only a single or a few tubers so that the

mass propagation is limited using this technique. Once tubers are infected by a virus, it will be difficult to be eliminated and transmitted to later generations through tuber (Myouda *et al.*, 2005).

The most common virus infecting *Dioscorea* is Yam Mosaic Virus (YMV) which belongs to the genus *Potyvirus* and is transmitted by aphids and through tubers (Thouvenel & Fauquet 1979). Another virus infecting *Dioscorea* is Yam Mild Mosaic Virus (YMMV), is also a member of genus *Potyvirus* but has a different host range and is serologically distinct from YMV (Mumford & Seal 1997). *Potyvirus* is the largest of six genera in the family Potyviridae and contains about 15% of all named plant virus species including BCMV or Bean Common Mosaic Potyvirus (Gibbs *et al.,* 2008). Yam mosaic virus (YMV) is one of diseases commonly found in *Dioscorea* species causing the reduction of its yield (Myouda *et al.*, 2005).

Virus-free plants is ussualy identified by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) and ACP-ELISA (Antigen Coated Plate Enzyme Linked Immunobsorbent Assay) as well as detection of YMV in *Dioscorea* (Myouda *et al.,* 2005; Malaurie *et al.,* 1998; Mumford & Seal 1997). RT-PCR is reported more accurate and sensitive method even for the symptomless plant samples or having low concentration of virus (Myouda *et al.,* 2005; Kajihara *et al.,* 2009). ACP-ELISA as a one of serological techniques have been extensively applied because of many advantages such as simple, faster and cheaper method than RT-PCR (Malaurie *et al.,* 1998).

Micropropagation and *in vitro* conservation of the genetic resources of *Dioscorea* is needed to be investigated in order to conserve their genetic diversity since up to now these species seem to be neglected. Establishing *in vitro* germplasm collection is a process to transfer plant collection to the *in vitro* environment (aseptic environment, free from pest, disease and include virus). This method is more useful compared to the conventional *ex vitro* conservation technique (Balogun 2009). Short-term conservation of *Dioscorea* germplasm is conducted by subculturing of the shoots cultures. Short-term conservation allows direct acclimatization of the shoot. *In vitro* propagation of many *Dioscorea* species are required to support shoots cultures collection subjected for the *in vitro* conservation (Klu 2002; Malaurie *et al.*, 1993; Malaurie *et al.,* 1998). An efficient shoots multiplication medium is investigated using MS basal medium containing cytokinins with various concentrations. Cytokinins are kind of plant growth regulator that support shoot proliferation by released axillary buds from apical dominance (Bhojwani & Razdan 1996).

The objective of this research was to detect *Potyvirus* using RT-PCR and ACP-ELISA for *D. alata*, *D. hispida* and *D. esculenta* in order to conduct an *in vitro* culture of virus-free *Dioscorea*.

## Materials and Methods

**Detection of *Potyvirus* using RT-PCR.** Materials used in this experiment were leaves of eight genotypes from three different species of *Dioscorea* e.i. *D. alata* (5 genotypes : white tuber collected from Bogor; white and purple tubers collected from Jogyakarta; purple tuber collected from Lampung; and purple tuber collected from Pasuruan), *D. esculenta* collected from Jogyakarta; and *D. hispida* also collected from Jogyakarta. All plants were grown in a greenhouse. Total RNA of eight plant genotypes grown in the glasshouse were isolated according to Simple Direct Tube (SDT) method (Suehiro *et al.,* 2005). Total RNA from symptomatic leaf of Yard Long Bean (*Vigna unguiculata*) infected with Bean Common Mosaic Potyvirus (BCMV) was used as the positive control treatment because BCMV is one of the members of genus *Potyvirus*. *Potyviruses* detection by RT PCR was conducted at Laboratory of Plant Virology, Departemen of Plant Protection, Bogor Agricultural University. cDNA amplification was performed using degenerate primers MJ1(F); 5'-ATGGTHTGGTGYATHGARAAYGG-3' and MJ2(R); 5'-TGCTGCKGCYTTCATYTG-3', designed by Marie-Jeanne *et al.,* (2000). RT PCR mix composition used was Go green Taq Premix PCR (Promega), RNA with final concentration 100 ng-5μg, and the primers with final concentration 10 μM/μl. The RT PCR condition was pre denaturation at 94ºC for 2 min; denaturation at 94ºC for 30 sec; annealing at 50 ºC for 1 min; extension at 72ºC for 1 min for 35 cycles and final extension at 72ºC for 10 min. PCR products were separated

on a 1% agarose gel with TBE buffer, for 45 min at 50 voltage. After electrophoresis the agarose gel was stained with ethidium bromide and visualized under UV light.

**Detection of *Potyvirus* using ACP-ELISA.** This experiment used five different plant genotypes as plant materials. Samples used were leaves of white tuber *D. alata* collected from Bogor and Jogyakarta; leaves of *D. esculenta* collected from Jakarta and Jogyakarta; and leaves of *D. hispida* collected from Jogyakarta. All plants were grown in a greenhouse. ACP-ELISA with *Potyvirus* genus-specific antibodies (AS-573/1; DSMZ, German Resource Center for Biological Material, Braunschweig, Germany) was carried out to detect *Potyvirus* from leaf extracts in PBS-Tween buffer. The absorbance was read at 405 nm using microtitre plate reader. Means for test samples and healthy control in duplicate wells were calculated. Based on the mean absorbance values obtained, each sample was scored positive or negative for *Potyvirus*. A sample was considered as infected (positive) when its absorbance was at least twice of that of the healthy control (Sutula *et al.*, 1986).

**Shoot culture of virus-free species *Dioscorea.*** Establishment of the shoot culture experiment of *Dioscorea* was conducted using seven different plant genotypes e.i. *D. alata* white and purple tubers collected from Jogyakarta; and purple tubers collected from Lampung and Pasuruan; *D. esculenta* collected from Jogyakarta and Jakarta; and *D. hispida* collected from Jogyakarta. All plants were grown in a greenhouse. Micropropagation of *Dioscorea* was conducted using lateral buds or nodal cuttings from *in vitro* culture as explants. The culture medium used was MS medium (Murashige & Skoog 1962) as the basal medium containing 30 g/l of sucrose, supplemented with 0.1; 0.5; 1.0 and 2.0 mg/l of cytokinin 6-benzylaminopurine (BAP). Medium without addition of BAP was used as the control treatment. The medium culture was solidified with 8 g/l of agar. Medium was sterilized using autoclave at 120$^{o}$C for 15 min. All cultures were maintained in a culture room at temperature of 26-27$^{o}$C using continuous light. Response of growth was observed after 5, 8 and 10 weeks of culture, this response was also recorded 4 months after culture. The growth response of explants recorded were height of shoots, number of nodes per explants, number of axillary shoots, and the formation of callus.

## Results and Discussion

**Detection of *Potyvirus* using RT PCR**

The infection of three species of *Dioscorea* e.i. *D alata, D. esculenta and D. hispida,* collected from several areas in Indonesia was assessed for *Potyvirus* by RT PCR. RT-PCR method was suitable to identify a viral RNA such as *Potyviruses*. Viral RNA needs to change to cDNA form with d(T)20 and MJ2 primer. In this research, MJ1 and MJ2 primers were used as primers for genus *Potyvirus*. The advantages in using these general primers are able to utilize total RNA from symptomatic leaf of Yard Long Bean (*Vigna unguiculata*) infected with Bean Common Mosaic Potyvirus (BCMV) from a previous study at Laboratory of Plant Virology, Departemen of Plant Protection, Bogor Agricultural University, was used as the positive control treatment.

Product RT PCR of *Potyvirus* was detected by the appearance of band with size of 327 bp (Figure 1). *Potyvirus* was not detected in any tested samples except for the control positive. MJ1 and MJ2 primers amplify a 327 bp fragment spanning conserved motifs MVWCIEN to QMKAAA in the core of the CP (Coat Protein) of potyviruses (Grisoni 2006). This result showed that all samples were not infected by *Potyvirus* where YMV is clasified in this virus class. Therefore, all *Dioscorea* samples could also be assumed to be free from YMV virus.

RT-PCR method was also reported to be used for *D. alata* (Myouda, 2005), *D. japonica* Thunb., *D. opposite* Thunb and *D. alata* L. (Kajihara, 2009) to detect the Japanese Yam Mosaic Virus (JYMV) as well as for the Yam Mild Mosaic Virus (YMMV) in yam plants in Japan. JYMV and YMMV are two virus species in the genus *Potyvirus*. These two virus are known to caused disease in yam plants (*Dioscorea*).

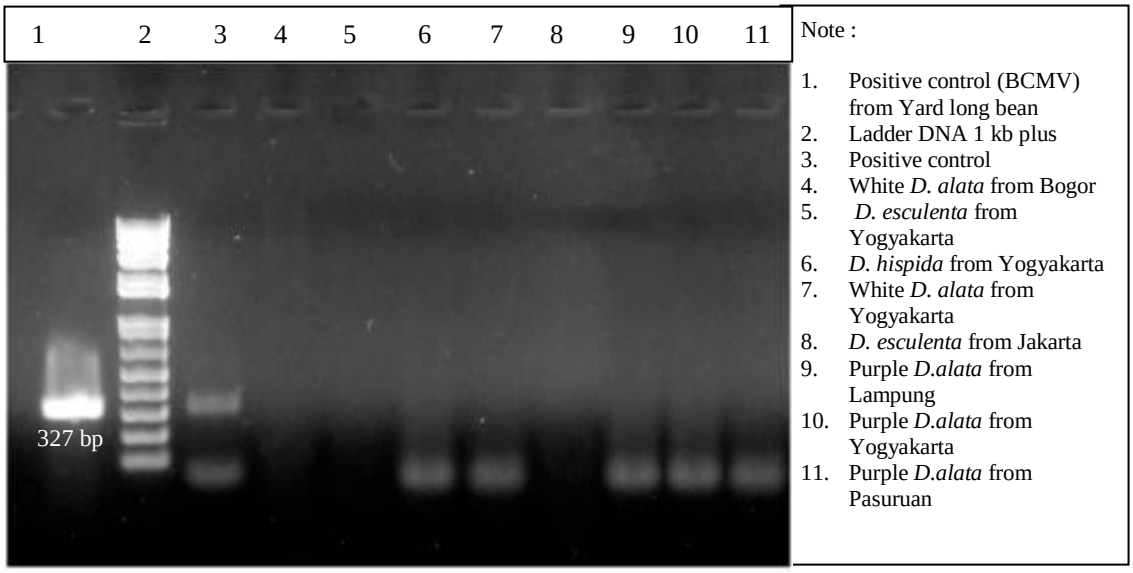


**Figure 1.** Detection of *Potyvirus* using RT-PCR for some species of *Dioscorea* using *Potyvirus* primer MJ1 and MJ2

**Detection of *Potyvirus* using ACP ELISA**

Detection of *Potyvirus* with ACP-ELISA technique was shown at Table 1, which is shown the absorbance value from a yellow water-soluble enzymatic reaction product that absorbs light at 405 nm wavelength. The 0.236 value was twice from absorbance value of negative control (2 x 0.118). The absorbance values of 5 *Dioscorea* genotypes from 3 different species (*D. alata, D. esculenta* and *D. hispida)* were ranging from 0.094 to 0.215. These values were less than 0.236, eventhough the absorbance value of *D. esculenta* from Jakarta was close to 0.236. This result coresponded with the result of the RT PCR product which showed that all *Dioscorea* collection was free from *Potyvirus*. In order to detect and to identify the specific plant viruses, serological tecniques such as the ELISA method may be applied because of this effectiveness even if in field conditions. However, among some viruses in the identical genus or among some strains of a virus, it is not always easy to distinguish among them because they may have very close serological relationship. This may cause difficulty in the detection of the spesific targeted virus. Malaurie *et al.,* (1998) also used ELISA method to controlled production of virus-free *Dioscorea* plants. They found that this method was easier and simpler to be applied. Four viruses were fetched by ELISA technique: PVX (Potato Virus X, potexvirus), PVY (Potato Virus Y, potyvirus), CMV (Cucumber Mosaic Virus, cucumovirus), and YMV.

**Table 1**. Absorbance value of *Dioscorea* samples detected by ELISA in 405 nm wavelenght

| Sample | Absorbance Value | Detection Result |
|---|---|---|
| Negative control | 0.118 | |
| Positive control | 1.874 | + |
| White *D. alata* from Bogor | 0.134 | - |
| White *D. alata* from Yogyakarta | 0.104 | - |
| *D. esculenta* from Yogyakarta | 0.117 | - |
| *D.esculenta* from Jakarta | 0.215 | - |
| *D. hispida* from Yogyakarta | 0.094 | - |

Note: Result was positive when samples absorbance value more than twice from absorbance value of control negative

*Dioscorea* plant species in Indonesia have not been developed as a staple food crop, cultivation in large area has not been done extensively. Detection of the YMV or other potyvirus infection on *Dioscorea* species potential for a carbohydrate source will contribute to other research done on this species. Micropropagation and *in vitro* conservation of virus-free *Dioscorea* are also required to overcome the excessive exploitation in the future.

**Shoots culture of virus-free species *Dioscorea***

Growth response of *Dioscorea* shoots cultured on MS medium is shown on Table 2 and Figure 2. MS medium without addition of

plant growth regulators only supported the primary growth of explants. The main shoot did not form axillary shoots. The nodes elongated with single leaf grew on each node. Cytokinin BAP added to the basal medium supported the growth of axillary buds.

In purple *D. alata* from Yogyakarta, the addition of BAP at low concentrations (0.1-0.5 mg/l) induced 2-7 axillary shoots growth and at higher concentrations (1.0-2.0 mg/l) supported more shoots ranging from 4 to 7 axillary shoots. The axillary shoots formed on the medium containing 1.0-2.0 mg/l of BAP were tiny than that of the normal shoots. The colour of shoots and leaves was purple at the beginning of growth. In the continuing growth, leaf blade become green with petiole remained purple, some parts of the main shoots were also remained purple. Some shoots developed into callus-like form.

In *D. esculenta* from Yogyakarta, addition of BAP at low concentrations (0.1-0.5 mg/l) induced 2-3 axillary shoots formation, BAP at high concentration (1.0-2.0 mg/l) supported 3-10 axillary shoots growth, but the size of shoots were smaller than normal shoots, some of them formed callus-like structure. It seems that BAP, as a synthetic cytokinin was stable, and effective for the induction of axillary shoots of *Dioscorea*.

Cytokinins are very effective in promoting direct or indirect shoot initiation and reduce apical dominance of main shoot (George & Sherrington 1984). MS basal medium without any growth regulator was suitable for short time *in vitro* conservation for *Dioscorea.* For shoot multiplication, MS medium containing 0.5-1.0 mg/l of BAP was suitable for *Dioscorea*. Chu (2002), resulted information that BAP also supported the higest formation of apical shoot on *D. delicata*. *D. olfersiana*, and *D. bulbifera* at 22 µM (equal to 5.0 mg/l) concentration. BAP also suitable for regenerating and multiplicated *D. alata* germplasm maintained *in vitro* (Borges, 2004).

In this research, shoot culture of *D. hispida* on MS0 medium easily to be browning. Ascorbic acid needed as antioxidant to reduce browning on medium. Behera *et al.*, (2008) obtained the highest number of multiple shoot proliferation with explants cultured on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA + 100 mg/l ascorbic acid. Adil *et al.*, (2003) multiplicated *D. hispida* for Bogor and Kuningan accessions in the WPM and Anderson medium enriched with $GA_3$, BAP and Thidiazuron.

## Conclusions

Using RT-PCR and ACP-ELISA, four species of *Dioscorea* was detected as free from *Potyvirus*, so that these plants can be used as healthy explants for micropropagation and for *in vitro* conservation. The growth response of shoots explants on MS medium was varied depending on the genotypes and concentration of BAP added to the culture medium. The *in vitro* culture of these species is useful for micropropagation as well as for *in vitro* conservation, so that the diversity of *Dioscorea* species will be available all over the time.

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**Table 2**. Growth response of *Dioscorea* species cultured on MS medium

| No | Species | Collected from | Growth response |
|---|---|---|---|
| 1 | White *D. alata* | Yogyakarta | On MS medium without growth regulators; after 5 weeks of culture; one main shoots with 2-6 nodes/explants |
| 2 | Purple *D. alata* | Lampung | On MS medium without growth regulators; after 5 weeks of culture; one main shoots with 1-2 nodes/explant |
| 3 | Purple *D. alata* | Pasuruan | On MS medium without growth regulators; after 5 weeks of culture; one main shoots with 2-3 nodes/explants |
| 4 | Purple *D. alata* | Yogyakarta | • On MS medium without growth regulators ; after 5 weeks of culture; one main shoots with 2-4 nodes/explant<br>• On MS+0.1 mg/l of BAP; after 5 weeks of culture; shoots explants formed 2-7 axillary shoots (5-20 mm height)<br>• On MS+0.1 mg/l of BAP (subcultured from MS+0.5 mg/l BAP); after 5 weeks in culture; shoots formed 2-7 axillary shoots (5-20 mm height)<br>• On MS+0.5 mg/l of BAP; after 9 weeks, shoots formed 2-7 axillary shoots (3-30 mm height)<br>• On MS+1.0 mg/l of BAP; after 5 weeks of culture; medium were brown; one main shoots with 4-7 nodes/explants; callus-like form with very small shoots.<br>• On MS+2.0 mg/l of BAP; after 5 weeks of culture; one main shoot with 2-5 axillary shoots (3-15 mm in height); callus-like form with very small shoots<br>• On MS without growth regulators (subcultured from MS+0.1 mg/l of BAP); 8 weeks after culture; one main shoot with 2-3 nodes/explants |
| 5 | *D. esculenta* | Yogyakarta | • On MS medium without growth regulators ; after 5 weeks of culture; one main shoot/explant; (roset) no node<br>• On MS medium without growth regulators (subcultured from MS+0.1 mg/l of BAP); after 8 weeks of culture; main shoot did not develop; 2-3 axillary shoots/explant (5-20 mm height)<br>• On MS medium without growth regulators (subcultured from MS+2 of mg/l BAP); after 8 weeks of culture; main shoot did not develop; 1-2 axillary shoots/explant (5-20 mm height)<br>• On MS medium without growth regulators (subcultured from MS+1 mg/l of BAP); after 10 weeks of culture; main shoot did not develop; 4-6 axillary shoots/explant (3-15 mm height)<br>• On MS+0.5 mg/l of BAP; after 4 months of culture; main shoot did not develop; 3-8 axillary shoots/explant (3-15 mm height)<br>• On MS+1.0 mg/l of BAP; after 5 weeks of culture; one main shoot with 1-3 axillary shoots/explants (3-5 mm height)<br>• On MS+2 mg/l of BAP; after 5 weeks of culture; 1-3 main shoots with 3-10 axillary shoots/explants (3-5 mm height) |
| 6 | *D. esculenta* | Jakarta | On MS medium without growth regulators; after 5 weeks of culture; one main shoots/explants; rosset (stems did not develop, no nodes found) |
| 7 | *D. hispida* | Yogyakarta | On MS medium without growth regulators; after 5 weeks of culture; medium were brown; one main shoots with 0-2 nodes/explants |

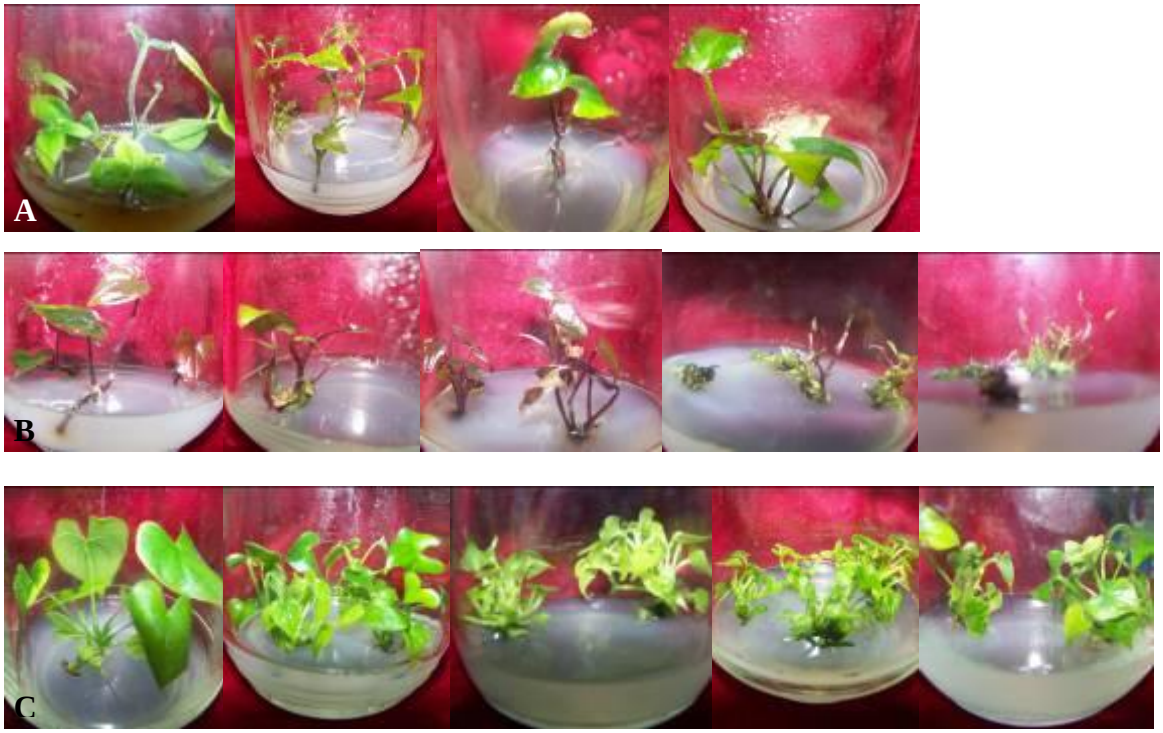


**Figure 2**. Shoot culture of *Dioscorea* grown on MS medium with and without addition of BAP. (A) *D. hispida*, white *D. alata* for Yogyakarta, purple *D. alata* from Lampung, purple *D. alata* from Pasuruan grown on MS medium, (B) Purple *D. alata* from Yogyakarta grown on MS, MS+0.1 mg/l of BAP, MS+0.5 mg/l of BAP, MS+1.0 mg/l of BAP, MS+2.0 mg/l of BAP media, (C) *D. esculenta* from Yogyakarta on MS, MS+0,1mg/l BAP, MS+0,5 mg/l BAP, MS+1.0 mg/l BAP, MS+2.0 mg/l BAP medium.

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