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BETTY CRISTIANE KUHN

Marcadores moleculares baseados em retrotransposons e marcadores EST-SSR para a caracterização genética e análise da variação somaclonal de Mandioca (*Manihot esculenta* Crantz)

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Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do titulo de Doutor.

Orientadora: Profª Drª Maria de Fátima Pires da Silva Machado.

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DEDICATÓRIA

Aos meus pais, Vilson e Leonides Kuhn.
Aos meus irmãos, Sama e Fagner.
Especialmente, ao meu esposo, Luiz Carlos Wessler, meu companheiro para todas as horas.

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BIOGRAFIA

Betty Cristiane Kuhn, filha de Vilson Kuhn e de Leonides Maria Kuhn, nasceu na cidade de Medianeira, estado do Paraná, no dia 03 de fevereiro de 1988.

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SUMÁRIO

RESUMO

KUHN, Betty Cristiane. Universidade Estadual de Maringá, março de 2015. **Marcadores moleculares baseados em retrotransposon IRAP e REMAP e marcadores EST-SSR para a caracterização genética e análise da variação somaclonal de Mandioca (*Manihot esculenta* Crantz).** Orientadora: Maria de Fátima Pires da Silva Machado. Conselheiros: Claudete Aparecida Mangolin e Eliezer Rodrigues de Souto.

A proposta no presente estudo foi estabelecer as condições adequadas para a multiplicação clonal de variedades de mandioca (*Manihot esculenta* Crantz) de interesse agronômico e industrial, indexadas para o Vírus Comum do Mosaico da Mandioca (CsCMV), e monitorar a estabilidade genética dos clones usando marcadores moleculares. Para a obtenção dos clones, foram utilizados meristemas apicais de sete variedades de mandioca (Baianinha, Fécula Branca, Icaraíma, IPR União, Olho junto, Pioneira e Tamboara) inoculados em meio de cultura com tipos e concentrações diferentes de auxinas e com concentrações diferentes de 6-benziladenina e ácido giberélico. Os clones das diferentes variedades foram estabelecidos em meios de cultura suplementados com diferentes tipos de auxinas. Para a indexação de vírus, foram usados os testes PTA-ELISA e IC-RT-PCR (*Immunocapture of CsCMV virions*). Para monitorar a estabilidade genética, foram desenhados 12 *primers* baseados em LTR (Long Terminal Repeat) de retrotransposon: IRAP (*Inter-Retrotransposon Amplified Polymorphism*) e REMAP (*Retrotransposon-Microssatellite Amplified Polymorphism*) os quais se mostraram adequados para analisar sequências de DNA das variedades Olho Junto, Fécula Branca e IPR-União. Os *primers* para IRAP e REMAP foram utilizados juntamente com *primers* para sequências simples repetidas contidas em sequencias expressas de DNA (EST-SSR) do genoma de mandioca, para investigar a possibilidade de indução de variação somaclonal nos clones. O polimorfismo nas sequencias de DNA amplificadas com os *primers* para IRAP e REMAP foi três vezes maior que o polimorfismo detectado com os *primers* EST-SSR nos clones das variedades Fécula Branca, Olho Junto e IPR-União; a variabilidade genética foi maior com o aumento do número de subcultivos. Os *primers* para IRAP e REMAP desenhados no presente estudo podem ser recomendados para monitorar a estabilidade em clones de mandioca propagados *in vitro*.

Palavras-chave: meristema apical, IRAP, REMAP, clonagem.

ABSTRACT

KUHN, Betty Cristiane. Universidade Estadual de Maringá, Maringá PR Brazil, March 2015. **Molecular markers based on retrotransposon IRAP and REMAP and EST-SSR markers for the characterization and analysis of somaclonal variations of Cassava (*Manihot esculenta* Crantz).** Adviser: Maria de Fátima Pires da Silva Machado. Committee Members: Claudete Aparecida Mangolin and Eliezer Rodrigues de Souto.

Current study establishes appropriate conditions for the clonal multiplication of cassava varieties (*Manihot esculenta* Crantz) with agronomic and industrial interest, free of the Cassava Common Mosaic Virus (CsCMV), and monitors the genetic stability of clones by molecular markers. Meristem-tips from seven cassava varieties (Baianinha, Fécula Branca, Icaraíma, IPR União, Olho junto, Pioneira e Tamboara) were employed to obtain the clones. They were inoculated in culture medium with different concentrations, types of auxin and different concentrations of 6-benzyladenine and gibberellin. The clones of the different varieties were established in culture media with different auxins. Texts PTA-ELISA and IC-RT-PCR (Immunocapture of CsCMV virions) were used for virus indexing. Twelve primers based on the LTR (Long Terminal Repeat) of retrotransposons were designed to monitor genetic stability: IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon-Microssatellite Amplified Polymorphism), suitable for analyzing the DNA sequences of varieties Olho Junto, Fécula Branca, Tamboara and IPR-União. The REMAP and IRAP primers were employed with primers to a simple sequence repeat contained in expressed sequences of DNA (EST-SSR) in the cassava genome, to investigate the possibility of induction of somaclonal variation in the clones. The polymorphism in DNA sequences, amplified with primers IRAP and REMAP, was more than three times greater than the polymorphism detected by the EST-SSR primers in clones of the varieties Fécula Branca, Olho Junto and IPR-União. Genetic variability was greater with the increasing number of subcultures. IRAP and REMAP primers designed for current study may be recommended to monitor the stability propagated in *in vitro* cassava clones.

Keyworks: Meristem-tip, IRAP, REMAP and cloning.

1. INTRODUÇÃO

A mandioca (*Manihot esculenta* Crantz) é uma importante cultura utilizada na alimentação por milhões de pessoas (Raemakers et al., 2001). É uma importante fonte de calorias, característica decorrente do elevado teor de amido das raízes (Mejía-Agüero et al., 2012) e as raízes tuberosas são utilizados para o consumo humano, ração animal e matéria-prima para a indústria de amido (Wongtiem et al., 2011). A mandioca representa a quarta maior fonte de calorias, depois do arroz (*Oryza sativa*), cana de açúcar (*Saccharum* spp.) e milho (*Zea mays*) (Medina et al., 2007). O baixo teor de proteína das raízes de mandioca a caracteriza como uma fonte de energia com baixo potencial alergênico (Ceballos et al., 2006).

A mandioca é multiplicada principalmente por estacas (manivas). Este é um processo lento, quando comparado com cultura de grãos (Santana et al., 2009) e a cada ano são produzidas estacas para plantar uma área oito vezes maior do que a de sua origem (Oliveira et al., 2000). O ciclo da cultura depende das variedades, se são precoces ou tardias. As primeiras são colhidas oito meses após o plantio, enquanto as tardias são colhidas após dezoito meses (Santana et al., 2009). A cultura da mandioca é afetada por várias doenças, especialmente as sistêmicas (Oliveira et al., 2000).

A perda da safra é agravada em virtude da mandioca ser propagada vegetativamente, devido ao plantio de material de qualidade inferior, ou à propagação de doenças (Bull et al., 2011). Na região noroeste do Paraná (Brasil), por exemplo, o vírus do mosaico comum da mandioca (CsCMV) infecta mais de 90% das plantas de todas as cultivares de mandioca (Silva et al., 2011).

A cultura *in vitro* de meristemas apicais de mandioca tem sido considerada um importante instrumento que permite a produção de uma grande quantidade de plântulas em um curto período de tempo e em espaço reduzido, possibilitando a produção de plantas livre de fungos, bactérias, vírus e parasitas (Roca et al., l989). Além disso, um sistema de regeneração eficiente também é essencial para o desenvolvimento de diversos processos, como a modificação genética (Raemakers, 2001) e a propagação de novas cultivares. Para conferir a limpeza de vírus em plantas cultivadas *in vitro*, podem ser aplicados métodos de detecção de vírus, sensíveis e rápidos, para a indexação das plantas. Variações de ELISA (*Enzyme*-

Linked Immunosorbent Assay) são utilizadas para detectar a presença de vírus e melhorar a sensibilidade de detecção do vírus, um protocolo de imunocaptura – RT-PCR (IC-RT-PCR) desenvolvido por Silva et al. (2011). Este método pode detectar o vírus CsCMV, mesmo em plantas consideradas livres de vírus pelo teste de ELISA.

Há necessidade de adaptação e adequação das condições de cultivo *in vitro* para os diversos genótipos de mandioca disponíveis. Feitosa et al. (2007) obtiveram resultados diferentes ao avaliar a frequência de embriogênese somática e número de embriões por explante em duas variedades de mandioca cultivadas no mesmo meio de cultura. Oliveira et al. (2000) também apontam que cada genótipo testado apresentou um efeito significativamente diferente durante o desenvolvimento *in vitro*.

Além das respostas diferenciadas das variedades de mandioca quando propagadas *in vitro*, o processo de cultura de tecidos pode gerar uma taxa de variabilidade em plântulas clonadas, variabilidade denominada variação somaclonal por Larkin e Scowcroft (1981). A variação somaclonal não é desejada na propagação comercial, quando se busca um grande número de plantas geneticamente idênticas (Bhojwani e Razdan, 1996) e, por isso, ela precisa ser identificada, monitorada e quantificada.

Uma das formas de identificar e quantificar as variações somaclonais é usando marcadores morfológicos e/ou moleculares. Dentre os marcadores moleculares que podem ser usados para analisar a variabilidade genética dos organismos estão os locos formados por sequências simples repetidas (*loci SSR; Simple Sequences Repeats*), que são encontradas no genoma dos organismos. Os locos microssatélites são importantes como marcadores moleculares porque as sequencias de bases destes locos são frequentemente variáveis e apresentam um polimorfismo alto, pois existe variação no número de repetições. Devido a sua grande variabilidade, os *loci SSR* são sequências que podem ser utilizadas para comparar plantas supostamente com pouco polimorfismo de DNA intraespecíficos, como é caso dos somaclones com origem comum (Chin et al., 1996).

Outros marcadores moleculares que podem ser aplicados no estudo da variação somaclonal são o IRAP (*Inter-retrotransposon Amplified Polimorphism*) e REMAP (*Retrotransposon-microsatellite Amplified Polymorphism*), ambos marcadores baseados em polimorfismo de sequencias de retrotransposons (Kalendar et al., 1999). O polimorfismo nos locos SSR e dos marcadores IRAP e REMAP pode ser maior, na proporção em que há um aumento no número de

estágios de micropropagação para produção de mudas de uma e/ou outra variedade de mandioca.

Desta forma, o objetivo no presente estudo é avaliar a estabilidade genética de somaclones durante o processo de multiplicação *in vitro* e indexação para o Vírus Comum do Mosaico da Mandioca das mudas de três variedades de *Manihot esculenta,* destinadas ao plantio comercial, investigando o número máximo de subcultivos que podem ser realizados em cada variedade sem a ocorrência de variação somaclonal**.**

2. REVISÃO DE LITERATURA

2.1. Cultura da mandioca

A mandioca teve origem na América do Sul, onde foi domesticada há aproximadamente 8.000 anos (Bull et al., 2011). O gênero *Manihot* inclui aproximadamente 97 espécies, distribuídas entre os trópicos, do México até a Argentina. A maioria das espécies ocorre na América do Sul (80 espécies) e o restante na América Central e México (17 espécies) (Olsen, 2004).

Para um grande número de países (tropicais e subtropicais), a mandioca é o alimento predominante na dieta diária, como fonte de carboidrato (Ceballos et al., 2006). É também empregada na nutrição animal, sob forma de farelos obtidos da parte aérea. Na indústria, a raiz pode ser utilizada como matéria prima na produção de amido (McMahon et al., 1995).

Dados de 1996 demonstram que os países da África destacam-se pelo consumo de mandioca, com um consumo per capita elevado, por exemplo, de 333,2 kg/hab/ano, na República Democrática do Congo, 281,1 kg/hab/ano, na República do Congo, e 247,2 kg/hab/ano, em Gana. Na África o consumo superou o do Brasil com 50,6 kg/hab/ano e também o consumo per capita mundial de 17,4 kg/hab/ano (Souza e Fialho, 2003).

O Brasil ocupa a quarta posição na produção mundial de mandioca (23 milhõs de Toneladas), depois da Nigéria (54 milhões de toneladas), da Tailândia (29,8 milhões de Toneladas) e da Indonésia (24,1 milhões de toneladas) (Faostat, 2012). A mandioca é a quarta cultura mais produzida no Brasil (Quadro 1), com produção menor que a da cana de açúcar, que é utilizada principalmente para a produção de açúcar e álcool. A produção de mandioca também é menor que a produção da soja e de milho, utilizados como alimento humano e também para ração animal (IBGE, 2014).

Quadro 1 - Produção (em mil toneladas) das quatro culturas de maior produção no Brasil nos anos de 2010 a 2014. Fonte IBGE

	2010	2011	2012	2013	2014
Cana de açúcar	717.462	734.006	675.015	739.267	692.772
Soja (grãos)	68.756	74.815	65.706	81.700	86.443
Milho (grãos)	55.364	55.660	71.491	80.538	79.035
Mandioca	24.967	25.349	24.314	21.226	23.573

O plantio da mandioca deve ser feito no início da estação chuvosa, pois a umidade e o calor são essenciais para a brotação, enraizamento e estabelecimento das plantas no campo, preferencialmente em solo com boa drenagem (variando de franco-arenoso a argilo-arenoso), uma prática eficiente para acelerar a cobertura do solo é a associação do plantio de mandioca em linhas duplas, com uma planta que tenha crescimento rápido, como leguminosas ou gramíneas nas entrelinhas (Embrapa, 2015).

Em áreas secas ou zonas semiáridas, a cultura da mandioca produz bem, quando comparada com outras culturas utilizadas para alimentação, pois é estável e tolerante a estresses hídricos. O principal mecanismo para esta tolerância inclui sensibilidade dos estômatos, ao lado da capacidade de enraizamento profundo (El-Sharkawy, 2006). O estado mais importante na produção de mandioca é o Pará, com uma participação de 21,1%, enquanto o estado do Paraná ocupa a segunda posição com uma produção de 3.815.221 toneladas em 2014, somando 16,5% da produção Nacional. Na terceira posição está o estado da Bahia, com 8,6% da produção, e em seguida o estado do Maranhão, com 7,0% (IBGE, 2014).

O cenário nacional não teve grandes alterações nos últimos cinco anos (Quadro 2), assim como também o estado do Paraná (Quadro 3), a produção, a produtividade e a área colhida se mantiveram estáveis, não variando muito com o passar dos anos (IBGE, 2014).

Quadro 2 - Produção, produtividade e área colhida no Brasil no período de 2010 a 2014. Fonte IBGE

Período	Produção (toneladas)	Produtividade (kg/ha)	Área colhida (ha)
2010	24.524,318	13.720	1.787,467
2011	25.329,667	14.520	1.744,446
2012	23.425,884	13.744	1.785,863
2013	21.225,782	13.915	1.525,441
2014	23.572,920	14.683	1.605,497

As raízes de mandioca apresentam quantidade de proteína relativamente baixa quando comparada com outras fontes de energia, como milho e aveia. Esta baixa concentração de proteínas pode ser considerada um problema para as pessoas que tem a mandioca como alimento base, mas esta raiz apresenta

vantagens, como o baixo potencial alergênico, sendo uma fonte ideal de energia para crianças (Ceballos et al., 2006). A massa fresca e o número de raízes por planta são fatores que exercem grande influência no rendimento da cultura e também determinam o valor comercial da matéria prima no momento da comercialização (Barbosa et al., 2007).

Quadro 3 - Produção, produtividade e área colhida no estado do Paraná no período de 2010 a 2014. Fonte IBGE

Período	Produção (toneladas)	Produtividade (kg/ha).	Área colhida (ha)
2010	4.012,948	23.300	172.214
2011	4.179,245	22.681	184.263
2012	4.128,520	22.146	185.704
2013	3.865,600	23.929	161.543
2014	4.073,990	23.170	175.831

Todos os tecidos da mandioca, com exceção das sementes, contêm compostos cianogênicos. Este composto produz cianeto após a sua degradação enzimática, ajudando a planta a evitar os danos causados por pragas e doenças (Fan et al., 2011). Quanto ao teor de cianeto, as variedades podem ser consideradas amargas (alto teor de ácido cianídrico), utilizadas com fins industriais, ou doces (baixo teor de ácido cianídrico), que podem ser utilizadas para o consumo imediato. As variedades utilizadas para o consumo imediato também são classificadas como “de mesa” (Kouassi et al., 2010). Vários distúrbios de saúde estão associados ao consumo da mandioca, pela presença de cianogénios residuais (Fan et al., 2011). Assim, antes do seu consumo, para eliminar o resíduo de cianeto, estas variedades devem ser processadas, para degradação do ácido (Kouassi et al., 2010).

As variedades de mandioca de mesa devem apresentar boas qualidades culinárias, baixa toxicidade cianogênica e resistência à deterioração pós-colheita (Borges et al., 2002). A quantidade de cianeto deve ser inferior a 100 mg/Kg para que a variedade possa ser utilizada no consumo humano, sem industrialização (Bolhuis, 1954).

Para a alimentação humana, a mandioca pode ser preparada de diferentes maneiras. No período que antecedeu a Copa do Mundo no Brasil em 2014, por exemplp, o SEBRAE lançou um boletim sobre o Panorama do Mercado da

Mandioca, inclusive, sugerindo aos empresários a comercialização de derivados da mandioca nas cidades que sediaram jogos da Copa. A lista inclui a mandioca *in natura* frita, “chips” de mandioca, tapioca, sagu e pão de queijo, que é preparado com o polvilho azedo (SEBRAE, 2014).

O amido de mandioca também é importante para atividades econômicas não associadas com a alimentação, como a produção de álcool e a utilização nas indústrias têxteis e de papel (Mejía-Agüero et al., 2011). O amido é convertido em produtos diferentes, de acordo com os costumes e preferências locais (Kouassi et al., 2010), mas a produção de etanol (biocombustível) tem se destacado (Wongtiem et al., 2011). Eijck et al. (2014) listaram os países que já utilizam a mandioca na geração de biocombustível: na África, é utilizada em Moçambique e na Tanzânia; e na Ásia, é utilizada pela Tailândia, onde ela é convertida em Etanol. Outras aplicações recentes para a utilização da raiz da mandioca é a sua utilização em pesquisas, como suporte de catalisador, e utilização industrial, como adsorvente de produtos químicos perigosos em fase líquida ou gasosa, destacando-se na adsorção de fenol (Jin et al., 2015).

2.2. Pragas da cultura da mandioca

A cultura da mandioca está sujeita ao ataque de insetos, ácaros e outros patógenos, como fungos, bactérias e vírus, e para sua eliminação algumas práticas de manejo são indicadas pela Embrapa Mandioca e Fruticultura (Embrapa, 2011), com destaque para a obtenção de ramas para plantio em áreas não infestadas e seleção de manivas-semente para propagação das plantas saudáveis.

A produção é atingida por diversos fatores bióticos, como o ácaro verde, o crestamento bacteriano, a cochonilha da mandioca, a doença do traço marrom da mandioca e a doença do mosaico (CMD), que é um begomovirus (família Geminiviridae) que pode causar perdas de 28-40% da produção, correspondente a 20-49 milhões de toneladas (Bull et al., 2011). Também se destaca a antracnose, causada pelo *Colletotrichum gloeosporioides,* que pode causar de 12 a 31% de perda na produção (Pinweha et al., 2015), e a *Xantomonas axononpodis pv. Manihotis,* causadora da bacteriose (Lopez et al. 2004).

As doenças que afetam a cultura da mandioca são especialmente sistêmicas e podem ser transmitidas ao longo de gerações sucessivas (Oliveira et al., 2000).

Pelo menos 16 vírus diferentes foram reportados na cultura da mandioca (Calvert e Tresh, 2002), dentre eles, o vírus do mosaico comum (CsCMV), considerado o mais frequente e importante. Causador de danos de grande importância econômica (Soares et al., 2009), gera um impacto significativo sobre o rendimento das culturas, qualidade da raiz e sobre os custos econômicos de comercialização e processos industriais (Bull et al., 2011).

O CsCMV é um *Potexvírus* de partículas semiflexuosas e em forma de haste (Kitajima et al., 1965) e seu genoma consiste em um RNA de fita simples de senso positivo (Nolt et al., 1991). Os vírus do gênero *Potexvirus* não possuem vetor conhecido e, portanto, sua disseminação ocorre através de práticas culturais, no preparo das manivas e na propagação das estacas (Colariccio et al., 2009). As plantas infectadas com CsCMV apresentam sintomas de mosaico com áreas cloróticas e normalmente distorção foliar. Silva et al. (2011) apontaram que, na região noroeste do Paraná, 90 % das plantas de todas as cultivares de mandioca estão infectadas pelo vírus CsCMV.

A cultura da mandioca é normalmente propagada vegetativamente, por estacas de 15-30 cm, o que torna comum a propagação de doenças, por plantio de material de baixa qualidade ou infectado por pragas (Bull et al., 2011). As sementes são principalmente utilizadas em programas de melhoramento genético (El-Sharkawy, 2006).

2.3. Cultura de tecidos para micropropagação de mudas

A aplicação industrial da técnica de micropropagação começou no final dos anos 1960 e início dos anos 1970, com espécies de orquídeas e posteriormente, com outras plantas (Vainionpaa et al., 1989). Além de permitir a produção de plantas com ótima qualidade fitossanitária e livres de vírus, a propagação *in vitro* (micropropagação) de mandioca foi considerada mais rápida que a convencional. Na propagação clonal convencional da cultura da mandioca, a razão de produção é de no máximo dez plantas, a partir de uma a cada 12 meses, e *in vitro* a razão é de cinco plantas a partir de uma, a cada seis semanas (Souza et al., 2008). A micropropagação *in vitro* é realizada com aplicação de três técnicas principais: cultura de meristema, embriogênese *in vitro* e indução de brotações em calos,

seguido pelo enraizamento, possibilitando a obtenção de plântulas clonadas em larga escala (Neumann et al., 2009).

Atualmente, o principal meio de cultura utilizado é o MS (Murashige e Skoog, 1962), com uma série de adaptações para fins específicos (Neumann et al., 2009). Diversas são as substâncias ou suplementos, conhecidos como “suplementos indeterminados”, que podem ser adicionados ao meio de cultura, tais como: água de coco (endosperma líquido de *Cocus nucifera*), carvão ativo, polpa de frutas, em especial a banana, dentre outros. Mas há também os “suplementos determinados”, por exemplo, os reguladores de crescimento vegetal, como as auxinas e citocininas (Dixon, 1985). De acordo com as considerações registradas por Soares et al. (2009), a composição e a concentração de reguladores de crescimento no meio de cultura são fatores que determinam o crescimento e o padrão de desenvolvimento da maioria dos sistemas de cultura de tecidos *in vitro*.

Os reguladores de crescimento são substâncias que atuam em vários processos do desenvolvimento das plantas em baixas concentrações. As auxinas desempenham importantes funções em quase todo o ciclo de vida da planta, como a divisão e a expansão celular, desenvolvimento de tecidos vasculares e o processo de indução de raízes laterais, quando utilizadas em concentrações adequadas, nos tecidos ou células de cada espécie aptas a receberem estes estímulos (Friml, 2003).

Para a micropropagação de plantas, a cultura de meristemas têm sido apresentada como adequada, produzindo plantas com ótima qualidade fitossanitária e livres de vírus. A proposição considera que as plantas obtidas por cultura de tecidos podem estar livres de vírus, pois a concentração de vírus não é uniforme e é menor em tecidos meristemáticos (explantes). Uma hipótese para explicar estas evidências é o fato de que o meristema apical ainda é avascular, o que impede a propagação do vírus (Guerra e Ronodari, 2006).

2.4. Variação somaclonal

A variabilidade gerada pelo processo de cultura de tecidos foi denominada de variação somaclonal, por Larkin e Scowcroft (1981). A partir da década de 80 do século passado, quando os referidos autores descreveram este tipo de variação gerada nas culturas *in vitro*, vários outros trabalhos vêm sendo feitos para demonstrar e quantificar as variações somaclonais em diferentes espécies de

plantas. Machado et al. (1999) observaram uma marcante e extensa variação na morfologia dos caules das plantas de *Cereus peruvianus* regeneradas a partir de tecidos de calos, que foi considerada como variação somaclonal. Santos e Rodrigues (2004) observaram diferenças na taxa de variação somaclonal em diferentes genótipos de bananeira, usando um mesmo protocolo de multiplicação, e mostraram diferenças de respostas dos genótipos ao cultivo *in vitro* e que, quanto maior o número de subcultivos, maior a variação somaclonal.

A variação somaclonal pode ser utilizada como ferramenta para geração de variabilidade, produzindo somaclones com características que não são observadas na natureza (Araújo e Prabhu, 2004). Assim, o uso da variação somaclonal tem sido útil na obtenção de genótipos mais tolerantes a estresses bióticos e abióticos (Barroso et al., 2003). Em arroz, por exemplo, a característica mais importante encontrada na variação somaclonal é a resistência a doenças. No Brasil, Araújo e Prabhu (2004) obtiveram quatro somaclones do Arroz CICA-8 com graus significativamente maiores de resistência parcial à brusone quando comparados à cultivar parental.

Mesmo apresentando algumas vantagens, a variação somaclonal pode não ser desejada quando a micropropagação tem como objetivo a produção de mudas geneticamente uniformes (Celédon et al., 2000). Para a propagação comercial de espécies arbóreas, a uniformidade genética também é um alvo e, neste caso, a variação somaclonal é considerada um fator negativo (Bhojwani e Razdan, 1996). A variação somaclonal gerada pelo cultivo *in vitro* pode acarretar em prejuízos aos produtores que procuram uniformidade para produção em larga escala (Silva et al., 2010).

2.5. Detecção da variação somaclonal com marcadores moleculares

O estudo da variação somaclonal é necessário, não apenas para detectá-la, mas também para acompanhar se as características desejáveis demonstram estabilidade nas gerações seguintes (Marshall e Courduries, 1992). A variação somaclonal tanto pode surgir a partir de fatores preexistentes na planta, como pode ser induzida durante a fase de cultivo *in vitro*. Essas variações podem determinar alterações no fenótipo e/ou genótipo dos indivíduos resultantes, podendo ser

detectadas a partir de análise isoenzimática e de outros marcadores moleculares (Skirvin et al., 1994).

Os marcadores morfológicos não se mostram confiáveis e também podem não ser viáveis. Em espécies arbóreas, por exemplo, o uso de marcadores moleculares para detectar variação somaclonal pode ser feito em estágios iniciais do desenvolvimento, enquanto para uma avaliação morfológica confiável seriam necessários muitos anos (Bhojwani e Razdan, 1996).

2.5.1. Locos de sequencias simples repetidas (SSR) como marcadores moleculares

Dentre os diversos marcadores moleculares disponíveis na literatura especializada para estudar o genoma de plantas, os locos de sequencias simples de DNA repetidas (locos SSR, também denominados locos microssatélites) têm sido apontados como um dos mais adequados. De acordo com os registros de alguns autores, a maior vantagem da análise dos microssatélites é o elevado polimorfismo revelado por estes marcadores, o que torna esta técnica uma das melhores opções para o uso na caracterização de variedades e cultivares, especialmente em germoplasma aparentado e com baixa variabilidade para outras características (Faleiro et al., 2001; Sereno et al., 2006; Lanaud et al., 1997).

Os microssatélites, ou repetições curtas em *tandem*, são repetições de 1 a 6 pares de bases (pb), distribuídas aleatoriamente através do genoma dos eucariotos (Jacob et al., 1991). As sequências de DNA que flanqueiam a região repetida dos locos SSR são conservadas e utilizadas para sintetizar *primers* (Schlötterer, 2000), os quais são então utilizados para a amplificação dos locos SSR. O polimorfismo é detectado quando existe diferença no comprimento do segmento após a amplificação destes por PCR. A identificação dos produtos é feita pela separação, por meio eletroforese, em géis de agarose de alta resolução, ou em géis de poliacrilamida corados com nitrato de prata. As regiões contendo sequências simples repetidas são amplificadas individualmente por meio de PCR, utilizando-se um par de *primers* específicos (de 20 a 30 bases), complementares às sequências que flanqueiam o microssatélite. Os segmentos amplificados dessa maneira apresentam polimorfismo extensivo como resultado da presença de diferentes números de elementos simples repetidos.

Os microssatélites são classificados em três famílias: de repetições puras, compostas e interrompidas. Nas repetições puras, os locos de microssatélites são formados por um único motivo repetido (5' TATTATTATTATTATTAT 3'); nas repetições compostas, mais de um motivo compõem o microssatélite (5' TATTATTATCACACACA 3'); e nas repetições interrompidas os motivos são intercalados por nucleotídeos que não fazem parte da unidade de repetição (5' CACATTCACACACATTCA 3') (Pinto et al., 2003a).

Usado amplamente como marcadores genéticos, os microssatélites possuem um atributo particular, pois sofrem elevadas taxas de mutação no genoma (Jarne e Lagoda, 1996). A variabilidade de tamanho observada nos locos de microssatélites, normalmente, deve-se à adição ou deleção do número de cópias das unidades repetitivas (Carvalho e Pitcher, 1994).

De acordo com Heywood e Iriondo (2003), os marcadores de microssatélite fornecem uma informação relevante, identificando unidades de conservação e investigando os processos genéticos que ocorrem nas populações, tais como testes padrões do fluxo de gene e de gerações geneticamente. Os marcadores de microssatélite são empregados, na maioria das vezes, para a análise da estrutura genética de populações de planta da mesma espécie (Zucchi et al., 2002) e de espécies selvagens (Pinto et al., 2003a,b). Devido à sua natureza codominante e por ser multialélico, um loco fornece um nível elevado de informação genética. Uma pesquisa mais recente baseada em sequencias *Tag* expressas (sequencias curtas de DNA clonados – cDNA, a partir de RNAm), frequentemente registradas como ESTs, sugeriu que a frequência dos microssátelites nas plantas é maior do que havia sido previsto (Morgante et al. 2002). O número de microssatélites é aproximadamente 1.844 no genoma de *Arabidopsis thaliana*, 2.757 no arroz, 2.000 na soja, 1.470 no milho e 1.796 no trigo.

Ramos et al. (2006) observaram que os marcadores microssatélites foram eficientes para determinar a pureza varietal de lotes de sementes de linhagens de milho, utilizados naquele estudo, com sensibilidade para detecção de concentrações de DNA iguais ou superiores a 0,01%, apresentando nitidez e repetibilidade, especialmente com a utilização de gel de poliacrilamida.

Bianchi et al. (2004) utilizaram a técnica baseada em marcadores de microssatélites em plantas de pessegueiro e nectarineira e concluíram que estes marcadores produzem elevado polimorfismo entre cultivares de pessegueiro e

podem ser utilizados na certificação da identidade genética das duas plantas cultivadas no Brasil.

Os microssatélites têm sido utilizados para estudos de diversidade e estrutura genética de populações, também aplicados para a conservação genética em *Oryza rufipogon* Griff. (Gao et al., 2000a, 2002a,b; Gao, 2004), em espécie de arroz selvagem de origem asiática reconhecida como ancestral da espécie cultivada *O. sativa* L. (Khush, 1997). Este marcador vem sendo também desenvolvido para outras espécies de arroz selvagem: *O. officinalis* Wall ex Watt., *O. granulata* Nees et Arn. ex Watt., *O. latifolia* Desv., *O. minuta* J.S. Presl. ex C.B. Presl., *O. australiensis* Domin e *O. ridleyi* Hook. (Ishii e McCouch, 2000; Gao et al., 2000b, 2005; Zhou et al., 2003). Os marcadores microssatélites têm sido empregados também com sucesso na obtenção de informação genética em variedades de mandioca (Silva et al., 2009; Vieira et al., 2009; Whankaew et al., 2011).

Apesar da grande variabilidade genética inter e intrapopulacional gerada pelo grande número de locos de microssatélite, a alta taxa de mutação pode, em alguns casos, evitar fixação de alelos dentro das populações. A presença dos mesmos alelos em diferentes grupos ou espécies pode diminuir a diferenciação interpopulacional ou interespecífica, quando comparado a outros tipos de marcadores (Fumagalli et al., 2002). Os marcadores SSR foram utilizados na análise da variação somaclonal de oliveira (*Olea maderensis* and *O. europaea ssp. europaea var. sylvestris*) (Brito et al., 2010), de pinheiro (P*inus pinea*) (Cuesta, 2010), de uva (*Vitis vinífera*) (Prado et al., 2010) e de algodão (*Gossypium hirsutum*) (Jin et al., 2008).

2.5.2. Sequencias EST na cultura da mandioca

Sequencias ESTs (*Expressed Sequence Tags*- Etiquetas de Sequências Expressas) funcionam como etiquetas de identificação de genes expressos em um determinado momento. Baseadas em sequências de cDNA geradas a partir de mRNA, estas sequências podem ser relacionadas a diversas respostas, como condições de estresse (Sakurai et al., 2007), resposta à seca (Lokko et al., 2007), resistência ou susceptibilidade à bacteriose e também produção de matéria seca (Lopez et al., 2004).

Prochnik et al. (2012) apresentam o progresso atual e os rumos esperados para o futuro dos trabalhos relacionados ao Genoma da Mandioca. Os autores destacam a base de dados (http://cassava.igs.umaryland.edu/blast/db/EST_ asmbl_and_single.fasta), na qual onde um conjunto de sequências EST de mandioca está disponível. As sequências de mRNA foram associadas a sequências de proteínas previamente identificadas em outras espécies e este alinhamento permite a análise comparativa da estrutura e função do genoma.

Coleções de ESTs foram geradas por Lopez et al. (2004) a partir de análises dos mRNAs expressos em diferentes cultivares de mandioca, relacionadas ao teor de amido e também à resistência ou susceptibilidade ao patógeno *Xanthomonas axonopodis*. O sequenciamento de ESTs de plantas deixou claro que muitas proteínas são codificadas por famílias de genes (caracteres quantitativos).

Uma biblioteca de cDNA de plantas de mandioca foi construída por Sakurai et al. (2007), a partir de plantas de mandioca submetidas a condições: normais, calor, seca, alta concentração de alumínio e condições de deterioração pós colheita. A partir destes dados, sequências ESTs foram caracterizadas e associadas a 7.796 genes distintos, possibilitando a classificação funcional de 78% destes genes, relacionando-os com enzimas já descritas para *Arabidopsis* e a identificação de 230 supostos genes (putative genes) duplicados e que apresentam papel importante na resposta da mandioca a estresse. Lopez et al. (2004) detectaram um conjunto de genes que se expressaram apenas nas cultivares inoculadas com o patógeno *Xanthomonas axonopodis* e identificaram que estes genes estão potencialmente relacionados à resposta de defesa.

Lokko et al. (2007) identificaram 18.166 sequências EST, que foram reduzidas a um total de 8.577 sequências UniGenes, divididas em duas bibliotecas: uma relacionada a plantas controle, bem regadas, e outra biblioteca relacionada a plantas sob estresse hídrico. Não apresentaram semelhanças com genes sequenciados anteriormente 25,71% das sequências ESTs e a maioria dos transcritos foi relacionada a genes associados ao metabolismo (10,19%) e à organização celular (8,69%).

Sequências ESTs foram associados à biossíntese de amido por Lopez et al. (2004) e várias isoformas de amido fosforilase e da enzima ramificadora 1,4-α-glucano foram caracterizadas a partir da coleção de genes de mandioca. Duas cultivares foram utilizadas para a identificação de sequências ESTs relacionadas ao

teor de matéria seca. As sequencias ESTs identificadas para a cultivar CM523-7 estavam relacionadas ao alto teor de matéria seca e as sequências EST identificadas a partir de mRNA da cultivar MPer183 estavam relacionadas com o baixo teor de matéria seca.

2.5.3. *Primers* EST-SSR

Kunkaew et al. (2011) obtiveram um total de 76.566 sequências ESTs a partir do banco de dados EST do NCBI (http://www.ncbi.nlm.nih.gov/dbest/). As sequências redundantes foram removidas pelos autores e reduzidas a 28.940 sequências EST únicas. Estas sequências únicas foram utilizadas para desenho de *primers* SSR e as sequências repetitivas também foram excluidas, resultando na identificação de 7.270 *primers* SSR, que foram utilizados por Kunkaew et al. (2011) para o mapeamento genético de mandioca. Dentre as sequências EST utilizadas como modelo para desenho dos *primers* EST-SSR, destacam-se: as sequências identificadas por Lopes et al. (2004), relacionadas à produção de matéria seca e resistência ao patógeno *Xantomonas*; as sequências relacionadas à resposta a estresse, identificadas por Sakurai et al. (2007); e as sequências relacionadas à resposta a seca, identificadas por Lokko et al. (2007).

Com o objetivo de caracterizar germoplasma de mandioca e possíveis aplicações no melhoramento genético, *primers* EST-SSR também foram desenhados por Raji et al. (2009b) baseados em sequencias EST identificadas por Lokko et al. (2007). Esses *primers* foram testados em cultivares de mandioca, provenientes da África, América e Ásia, assim como também de quatro espécies de mandioca selvagem e outras duas espécies da família das *Euphorbiaceae*. Um total de 124 *primers* EST-SSR, novos e exclusivos para o genoma da mandioca, foram desenvolvidos por Raji et al. (2009a) e caracterizados úteis para identificação de proteínas de interesse, principalmente, relacionadas à resposta ao estresse hídrico (Lokko et al., 2007).

Kunkaew et al. (2011) utilizaram *primers* EST-SSR em conjunto com marcadores SSR para a construção do mapa de ligação genética da mandioca, apontando que este estudo é útil para a identificação de locos de características quantitativas que controlam características de interesse em programas de melhoramento genético de mandioca. Sraphet et al. (2011) também utilizaram com

sucesso os marcadores EST-SSR em conjunto com *primers* SSR, para a construção de um mapa de ligação genética da cultura da mandioca.

2.6. Retrotransposons

Os retrotransposons compreendem uma família de sequências repetidas em *tandem* e dispersas no genoma das plantas. Algumas famílias de retrotransposons estão representadas por milhares de cópias no genoma e a maioria destes elementos está inserida aleatoriamente no genoma, sem produzir alterações genéticas nem fenotípicas. Devido a estas propriedades, os Retrotransposons têm sido utilizados com êxito como base de sistemas de análise da variabilidade genética (Kalendar et al., 2011).

Os retrotransposons são elementos dinâmicos e existem em alta porcentagem no genoma da maioria dos organismos, mas a maioria dos retrotransposons é inativa durante a fase de desenvolvimento e algumas condições de estresse estimulam a atividade de retrotransposons, como, por exemplo, as condições de cultivo *in vitro,* devido ao estresse nutricional, químico, físico e de fotoperíodo (Bayram et al., 2012).

Du et al. (2009) destacaram que os marcadores baseados em retrotransposons são uma importante ferramenta no estudo de instabilidade genética e evolução genômica. Em 1993, Hirochika publicou um trabalho pioneiro, no qual analisou elementos de transposição e constatou que eles se movimentam no genoma de *Nicotiana tabacum* durante o processo de cultivo *in vitro*. Esta foi a primeira demonstração da atividade de retrotransposons de plantas durante a cultura de tecidos. Bayram et al. (2012), analisando calos de cevada em diferentes idades (*Hordeum vulgare* L.), observaram que os retrotransposons do grupo *Nikita* também se movimentam pelo genoma, durante o processo, sendo uma ferramenta útil na análise da variação somaclonal. Bradley et al. (2011) também utilizaram um marcador molecular baseado em retrotransposon na detecção de variação somaclonal em cevada. Neste estudo, o marcador utilizado foi o IRAP e os autores afirmaram que este marcador oferece uma detalhada caracterização dos perfis de mutação apresentados durante a cultura de tecidos. De acordo com estes investigadores, o uso deste marcador pode ser útil no entendimento dos processos que influenciam a mutação e a variação somaclonal para o desenvolvimento de

métodos que não causem mudanças no genoma, em casos onde a sua integridade é importante.

Os elementos transponíveis mais abundantes nos eucariotos são os retrotransposons, os quais consistem em LTR (*Long Terminal Repeat*) e *non*-LTR. Os transposons LTR são divididos em LINEs (*long interpersed repetitive elements* e SINEs (*short interpersed repetitive elements*), que podem se apresentar em um número alto de cópias (mais de 250.000) nas espécies de plantas estudadas até agora. Os retrotransopsons de plantas são estruturalmente e funcionalmente similares aos retrotransposons e retrovírus de outros organismos eucariontes (Kumar e Bennetzen, 1999).

Um dos sistemas de análise molecular baseado em retrotransposons é o IRAP (*Inter-retrotransposon amplified polymorphysm* (Kalendar e Schulman, 2006), que consiste na amplificação por PCR de fragmentos de DNA entre cópias próximas a retrotransposons, utilizando um *primer* baseado na sequência LTR final de um retrotransposon, voltado para fora do mesmo, para amplificar até o retrotransposon seguinte. Desta maneira, são gerados uma série de fragmentos, cujo tamanho pode ser determinado por eletroforese. O uso de um número suficiente de *primers* gera centenas de bandas e os padrões de bandas podem ser utilizados para determinar as relações genéticas entre diferentes linhagens de uma mesma espécie (Kalendar et al., 2011). A eficiência desta técnica para determinar variabilidade genética foi demonstrada, pois tem sido utilizada com êxito em diferentes espécies vegetais, como cevada (Vicient et al., 2005), linho (Smykal et al., 2011), girassol (Vukich et al., 2009), ervilha (Smykal et al., 2008) e batata (Lightbourn et al., 2007).

Outro sistema de análise molecular baseado em retrotransposons é o REMAP (*Retrotransposon-Microsatellite Amplified Polymorphism*), método similar ao IRAP, mas que utiliza também um *primer* SSR, com um nucleotídeo não-SSR de ancoragem, presente na direção 3’ final do *primer* (Kalendar e Schulman, 2006).

Scowcroft (1985) aponta que a diferença no tempo de duração da cultura de tecido pode gerar um aumento no número de cópias de retrotransposons, que podem ser úteis para estudos sobre a variação somaclonal. Hirochika (1993) observou que células de *Nicotiana tabacum* perderam a totipotência após passarem muito tempo sendo cultivadas *in vitro*. O autor aponta que é possível que os genes associados para a regeneração podem ter sofrido mutações por elementos de transposição nestas linhas celulares. Campbell et al. (2011) utilizaram marcadores

IRAP e ISSR em cevada e constataram que marcadores baseados em retrotransposons são ferramentas valiosas para a caracterização detalhada de perfis de mutação que surgem durante a cultura de tecidos e seu uso permite a concepção de métodos de cultura de tecidos que produzam menos variação somaclonal.

Os marcadores IRAP e REMAP foram utilizados por Mandoulakani et al. (2012) para análise da diversidade entre e dentro de populações e genótipos de alfafa (*Medicago sativa*) para identificar populações com distâncias genéticas maiores e determinar grupos heteróticos para potencial aplicação em cultivares de alfafa e programas de melhoramento. Os autores obtiveram resultados satisfatórios com a análise dos retrotransposons, encontrando uma diversidade entre populações de 92% e dentro de populações de 8%, podendo, assim, eleger genótipos para programas de melhoramento.

Os marcadores IRAP e REMAP desenvolvidos por Kalendar et al. (1999) têm sido aplicados em cereais (Kalendar et al. 1999; Kalendar e Schulman 2006) cevada (Campbell et al., 2011) alfafa (Mandoulakani et al. 2012), milho (Kuhn et al., 2014) e também em fungo, como o shitake (*Lentinula edodes*) (Xiao et al., 2011).

Na literatura, há registro do estudo de retrotransposons na cultura da mandioca por meio dos marcadores basedos em MULE (*Mutator-like transposable element*) (Gbadegesin et al. 2007). Também há pesquisas sobre a análise da atividade dos elementos de transposição En/Spm-like de mandioca (Gabadegesin e Beeching, 2010), isolamento e caracterização de membros de duas classes de LTR-retrotransposons *Ty1/copia-like* e *Ty3/gypsy-like* e de *Enhancer/Suppressor Mutator (En/Spm)-like* (Gbadegesin et al., 2007) e também (*Inter-Retrotransposon Amplified Polymorphism*) realizado por Silva et al. (2014).

Gbadegesin et al. (2007) sequenciaram 154 retrotransposons do genoma da mandioca e disponibilizaram nos acessos AY946045 – AY946199, os quais foram classificados como membros de duas classes de LTR-retrotransposons, sendo 59 famílias *Ty1/copia* e 26 famílias de *Em/Spm* no genoma da mandioca. Gbadegesin e Beeching (2010) apontaram que os elementos de transposição contribuem com o tamanho, estrutura, variação e diversidade do genoma e exercem grande efeito na função dos genes. Os autores também destacaram que projetos de sequenciamento revelaram a diversidade dos elementos de transposição de muitos organismos e que estes representam uma alta porcentagem na constituição do genoma.

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CAPÍTULO I

AN IMPROVED SYSTEM FOR RAPID *IN VITRO* REGENERATION OF VARIETIES OF CASSAVA (*MANIHOT ESCULENTA*) RELATED TO GENETIC DIVERSITY

ABSTRACT

Current assay establishes an improved protocol to generate *in vitro* culture of commercial cassava cultivars (*Manihot esculenta*) and index the plants for CsCMV virus. An analysis of the relationship between the development of the cultivar *in vitro* and their genetic diversity was also established. Meristem-tip of seven varieties were inoculated in four concentrations of naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) in the MS culture medium. After the establishment of the meristem-tip, the plantlets were transferred to media supplemented with higher NAA and IBA concentrations containing gibberellic acid for faster development The Fécula Branca variety showed 100% of regenerated plants in the medium containing IBA, while the IPR-União variety showed 100% of regenerated plants in the medium containing NAA. The media containing IBA or NAA were equally effective for the Olho Junto variety. The genetic diversity detected among the seven varieties was used to explain the differential responses in the culture medium. Virus cleaning was efficient in the variety IPR-União with 70% of free-virus plants.

Keywords: *Manihot esculenta*, grown regulator, meristem-tip, virus, genetic diversity.

1. INTRODUCTION

Cassava (*Manihot esculenta*), an important food crop for millions of people worldwide (Raemakers, 2001), is the fourth greatest source of calories after rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) (Medina et al., 2007). The low protein content of cassava roots makes it a source of energy with low allergenic potential (Ceballos et al., 2006). Cassava is a perennial shrub of the family Euphorbiaceae (Wongtiem et al., 2011) native to South America (Oliveira et al., 2000), but grows in all tropical and subtropical regions (Fan et al., 2011). Cassava features easy propagation systems, satisfactory performance even in dry and low fertility soils, and low demands for sophisticated cultural requirements (Medina et al., 2007). Moreover, it provides a high return per unit of energy used in its cultivation (Mejía-Agüero et al., 2012).

Cassava is multiplied mainly by stem cuttings which is actually a slow process when compared with grain crops (Santana et al., 2009). The crop cycle depends on whether the early or late varieties are grown: the first type varieties are harvested eight months after planting, whereas the second type varieties are collected 18 months post planting (Santana et al., 2009), They may be affected by several diseases, especially systemic, which are transmitted through successive generations (Oliveira et al., 2000). Since cassava is vegetively propagated, the loss of crop is heightened due to the planting of suboptimal material, or by spreading diseases (Bull et al., 2011). The most frequent disease in Brazil is the CsCMV (Cassava Common Mosaic Virus) (Soares et al., 2010) which causes significantly impact on crop yields, root quality, economic costs, marketability, consumer availability and commercial processes (Bull et al., 2011). In fact, in the northwest region of the state of Paraná (Brazil), CsCMV infects more than 90% of plants from all cassava cultivars (Silva et al., 2011).

The *in vitro* tissue culture may be an alternative for the production of healthy cassava seedlings. The meristem-tip culture is an important tool which enables the production of a great deal of seedlings in a short time and in a reduced space, coupled to plants free of fungi, bacteria, viruses and pests (Roca et al., l989). Although the meristem-tip culture is a promising method to produce virus-free cassava plants, its application to new cultivars requires improved protocols since

different genotypes may have different developments in the culture media (Oliveira et al., 2000; Feitosa et al., 2007; Fan et al., 2011).

After the establishment of tissue culture, the generated clones should be indexed with virus detection methods. Variations of ELISA have been used to detect viruses and more recently a new method has been established to improve virus detection sensitivity. An immunocapture-RT-PCR (IC-RT-PCR) protocol was established by Silva et al. (2011) and has been reported to be more sensitive and a faster protocol than ELISA alone.

Current assay establishes an improved protocol for the *in vitro* generation of new high-yielding cassava cultivars of commercial and industrial interest and index the plants for CsCMV virus. The genetic relationship among the varieties inoculated in the culture media was also analyzed to see how much the different genotypes showed differential development in different culture media. The molecular marker based on retrotransposons LTRs (Kalendar et al., 1999; IRAP and REMAP) was employed to investigate the genetic relationship among the cassava cultivars. The Inter-retrotransposon amplified polymorphism (IRAP) and the Retrotransposon-micro-satellite amplified polymorphism (REMAP) markers are abundant in the cassava genome and produce a high number of reproducible DNA segments (Kuhn et al., 2015 unpublished data). It may be possible that the meristem-tip culture of cassava cultivars showing high genetic similarity may be established in culture medium with the same supplements.

2. MATERIALS AND METHODS

2.1. Selection of plant material

In current study, the cassava varieties (Baianinha, Fécula Branca, Icaraíma, IPR-União, Olho Junto, Pioneira and Tamboara) were obtained from the cassava germplasm bank maintained at the IAPAR (Agronomic Institute of Paraná, Brazil). Fresh stem cuttings measuring 20-30 cm, with 5-8 nodes, were cultivated in plastic trays and kept under greenhouse conditions at the Universidade Estadual de Maringá, Maringá PR Brazil.

Cassava leaves were harvested from plants in the greenhouse and submitted to PTA-ELISA analysis prior to *in vitro* propagation (Figure 1).

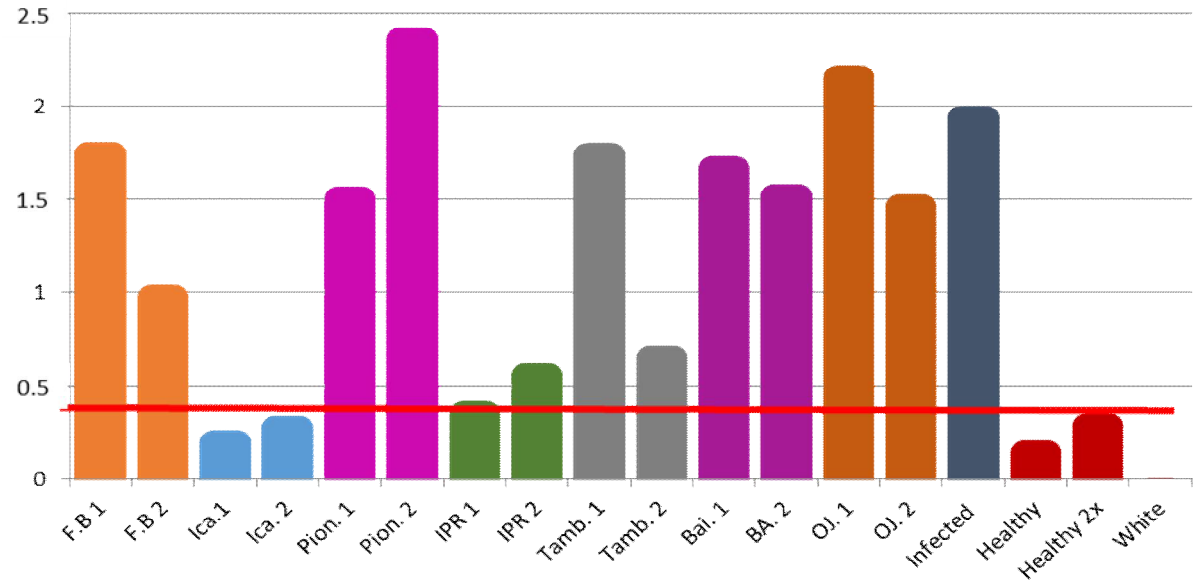

Figure 1 - PTA-ELISA (CsCMV virion) of cassava cultivated in the greenhouse prior to *in vitro* propagation. Cultivars: F.B: Fécula Branca; Ica: Icaraíma; Pion: Pioneira; IPR: IPR-União; Tamb: Tamboara; Bai: Baianinha; OJ: Olho Junto. **Infected** is one plant with virus previously indexing. Healthy is one the IAC 12 plant. (Each bar corresponds to one plant). The red line representes 2x the healthy value, the limmit of healthy plants, values greater than this are considered with vírus.

The CsCMV vírus is widespread in the cassava. Although the cultivar Icaraíma was not infected by the CsCMV, the virus infected the other six cultivars tested. Consequently all parental plants were infected by the virus and clonal

cleaning was required. The shoots harvested from the plants with the lowest virus index tested by PTA-ELISA were used for *in vitro* propagation (Figure 2).

Figure 2 - **A:** Cassava plants in the greenhouse. **B:** Cassava stem. **C:** Cassava shoot.

2.2. Plant material and culture establishment

Shoot tips (2.0 cm) were collected from the cassava plants and washed in distilled water with two drops of Tween 20 for 10 minutes. Sterilization was subsequently carried out in a laminar airflow cabinet under aseptic conditions, or rather, the shoots were sterilized by immersion in alcohol 50% for 1 minute, followed by 0.5% calcium hypochlorite for 5 minutes, and cleaning in sterilized water for three times during 2 minutes.

Meristem-tips with no leaf primordia (<0.4 mm) were aseptically dissected and transferred to test tubes (15 x 2.5 cm) containing 10 ml of solidified MS medium, supplemented with different concentrations of grown regulator (Table 1); the pH of all media was adjusted to 5.8 before adding agar and autoclaved for 15 min at 121 °C and 1.2 kPa. The tubes with explants were incubated at 26 ± 2 ºC within a 16-hour photoperiod provided by white fluorescent lamps (25.3 µmol m^{-2} s^{-1}).

Each treatment consisted of 10 explants (meristem tip) and the data were recorded three weeks after the beginning of the treatments. Data were analyzed according to the development and mortality rate of seedlings.

Table 1 - MS medium (Murashige and Skoog, 1962), supplemented with different concentrations (mg · L^{-1}) of **6-Benzylaminopurine** (6-BA), **Naphthaleneacetic acid** (NAA), **Indole-3-butyric acid** (IBA), **Gibberellic acid** (GA_3) and sucrose (%) for the establishment of cassava tissue culture

Medium	6BA	NAA	IBA	GA_3	Sucrose
C1	0.4	0.2	-	-	3%
C2	0.4	-	0.2	-	3%
C3	0.2	0.1	-	-	3%
C4	0.2	-	0.1	-	3%
C5	0.04	0.02	**-**	-	3%
C6	0.04	-	0.02	-	3%
C7	0.04	0.02	-	0.05	3%
C8	**0.04**	**-**	**0.02**	**0.05**	**3%**
$C9^7$	0.2	0.1	-	0.05	2%
$C10^8$	0.2	-	0.1	0.05	2%

$C9^7$ seedlings formed on C7 [which have a lower concentration of 6BA (0.04 mg L^{-1}) and NAA (0.02 mg · L^{-1}) to avoid callus formation] after 60 days were transferred to $C9^7$.
$C10^8$ seedlings formed on C8 [which have a lower concentration of 6BA (0.04 mg · L^{-1}) and IBA (0.02 mg · L^{-1}) to avoid callus formation], after 60 days were transferred to $C10^8$.

Growth was analyzed 60 days after inoculation. The meristem tip provided plantlets *in vitro* which were divided into 2 at 4 pieces (with side shoots) for fast multiplication. One mother plant generated between 5 and 15 cloned plants in each subculture, and each meristem tip was subcultured 5 times. After the five subcultures, the plantlets generated were transplanted to plastic cups with substrate Plant Max covered by cups to maintain humidity for 1 week. They were then taken to the green house and the cup was gradually removed, following Souza (2008). Ten regenerated plantlets were randomly selected for each cultivar and indexed for CsCMV virus.

2.3 PTA-ELISA virus detection

Young cassava leaves were harvested from plantlets grown from meristem-tip culture after 5 subculture cycles (plus an additional 60 days in greenhouse acclimatization) and 10 random plantlets for each line cultivated *in vitro* were tested. Conditions for PTA-ELISA analysis followed Mowat & Dawson (1987). Each sample was tested in triplicate wells of a polystyrene microtiter plate. Extracts from healthy

cassava cv. (IAC 12) plants were used as negative controls, whereas extracts from an infected cassava cv. Baianinha plant was used as positive control. Tests were considered positive when the absorbance (A405) rate of each sample was at least two times greater than that of the respective healthy control plant.

2.4. IC-RT-PCR detection

After the PTA-ELISA, the virus-free plants were also tested by IC-RT-PCR, a more sensitive method than ELISA. Immunocapture of CsCMV virions was performed according to protocol by Silva et al. (2001) with 50 µL of virus-specific IgG, incubated for 180 min at 37 ^{o}C and subsequently washed three times with PBS-Tween. Tubes were filled with 50 µL of extract and incubated at 6 ^{o}C overnight, followed by two washes with PBS-Tween and one wash with DEPC-treated water prior to RT-PCR. Complementary DNA (cDNA) was prepared in a final volume of 20 µL. Tubes were filled with 1 µL of random primers (500 ng), 2 µL of dNTPmix (10 mM) and 3 µL of DEPC-treated water, heated at 65 °C for 5 min and immediately chilled on ice. A mixture of 4 µL of 5× first strand buffer, 2 µL of DTT (0.1M), 1 µL of RNAse OUT (40 U) and 200 U of M-MLV were added to the tubes. They were heated at 25 °C for 10 min and then at 42 °C for 50 min. For the PCR, 5 µL of the cDNA was placed in a new tube, followed by 5 µL of 10× PCR Buffer (200 mM) Tris-HCl (pH 8.4), 500 mM KCl], 3 µL of $MgCl_2$ (50 mM), 2 µL of dNTP mix (10 mM), 2 µL of each primer (10 µM), 1 µL of *Taq* DNA polymerase (5 U) and 30 µL of DEPC treated water. The primer set used in this assay is universally used for members of the genus *Potexvirus* (Gibbs et al., 1998).

2.5. IRAP and REMAP amplification

Genomic DNA obtained from the parental varieties and used for clone regeneration *in vitro* was isolated with CTAB (cetyltrimethylammonium bromide) protocol (Knapp & Chandlee 1996), adapted by Carvalho (Cenargen – Embrapa). The genomic DNA was dissolved with TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and quantified with picodrop (Pico200 spectrophotometer).

PCR reactions were performed in 25 µL volume: 30 ng of DNA, 1xPCR buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 4 pmol of each primer, 100 µL dNTP, and 1 U of PrimeSTAR DNA polymerase (Takara Bio,

Tokyo, Japan). Amplification was performed in a MJ Research PTC-200 PCR Peltier Thermal Cycler (Bio-Rad, Hercules, USA). After 10 min of initial denaturation at 95 °C, amplifications were carried out in 36 cycles at 94 °C for 30 sec; at 55 °C for 30 sec and at 72 °C for 3 min, with a final extension of 10 min at 72 °C. The PCR products were identify by electrophoresis with 3% agarose (ECOGEN, AG-0600, Madrid, Spain) in 1xTAE buffer followed by EtBr staining and UV visualization. PCRs were repeated three times with similar results. The primer combinations (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5), designed and tested by Kuhn et al. (unpublished data), were used for DNA amplification. The amplified DNA segments were analyzed by comparing IRAP and REMAP profiles of each plant in terms of presence or absence of each DNA segment. Plants' similarity was calculated by Jaccard's coefficient, while UPGMA cluster analysis was performed with NTSYS-pc software (Rohlf, 1989).

3. RESULTS AND DISCUSSION

3.1. *In vitro* cassava propagation

After 60 days the meristems cultivated in the culture media C1 (0.4 mg L^{-1} of 6BA, 0.2 mg L^{-1} of NAA and 3% of sucrose) and C2 (0.4 mg L^{-1} of 6BA, 0.2 mg L^{-1} of IBA and 3% of sucrose) generated callus with no regeneration of planlets. In the culture media C3 (0.2 mg · L^{-1} of 6BA, 0.1 mg · L^{-1} of NAA and 3% of sucrose), C4 (0.2 mg L^{-1} of 6BA, 0.1 mg · L^{-1} of IBA and 3% of sucrose), C5 (0.04 mg L^{-1} of 6BA, 0.02 mg L^{-1} of NAA and 3% of sucrose), and C6 (0.04 mg L^{-1} of 6BA, 0.02 mg L^{-1} of IBA and 3% of sucrose) most regeneration plantlets die, whilst those that did not die had their growth impaired and presented a yellow color. The plantlets of the Fécula Branca, Icaraima, Pioneira and Tamboara varieties showed satisfactory growth in the C8 medium, whereas variety IPR União grew in the C7 medium and variety Olho Junto showed low growth in C7 and C8 culture media (Table 2).

Table 2 - Growth of plantlets from the different cassava cultivars in different culture media. R: Regenerated plantlets; C: Callus formation

	Cultivar						
Culture medium	**Baianinha**	**Fécula Branca**	**Icaraíma**	**IPR União**	**Olho Junto**	**Pioneira**	**Tamboara**
C1	60% C	100% C	100% C	80% C	100% C	100% C	100% C
C2	100% C	100% C	100% C	90% C	100% C	100% C	100% C
C3	60% R	70% R	60% R	80% R	90% R	90% R	90% R
	10% C	0% C	0% C	0% C	40% C	50% C	40% C
C4	80% R	80% R	80% R	80% R	80% R	90% R	60% R
C5	50% R	60% R	60% R	80% R	30% R	50% R	40% R
C6	60% R	50% R	40% R	60% R	20% R	40% R	30% R
C7	70% R	70% R	60% R	100% R	90% R	60% R	70% R
C8	60% R	100% R	90% R	80% R	100% R	80% R	100% R
C9[7]	--	--	--	100% R	100% R	--	--
C10[8]	--	100 % R	--	--	100% R	--	--

In the culture media C7 (0.04 mg · L^{-1} of 6BA, 0.02 mg · L^{-1} of NAA, 3% of sucrose, and 0.05 mg L^{-1} of GA_3) and C8 (0.04 mg· L^{-1} of 6BA, 0.02 mg L^{-1} of IBA,

3% of sucrose, and 0.05 mg · L^{-1} of GA_3), death rate was low (<30% and <20%, respectively). The plantlets grown in the culture media C7 and C8 were then transferred to culture medium with a higher concentration of cytokinin and auxins: 0.2 mg L^{-1} of 6BA and 0.1mg L^{-1} of NAA in the culture medium $C9^7$, and 0.2 mg L^{-1} of 6BA and 0.1 mg L^{-1} IBA in the culture medium $C10^8$, where the seedlings were kept growing for a further 30 days.

Although the culture media C7 and C8 were efficient to develop cassava meristems, higher concentrations of cytokinin and auxins in $C9^7$ and $C10^8$ culture media were needed to complete the *in vitro* development of seedling from the Fécula Branca, IPR-União and Olho Junto varieties. The varieties Icaraima, Pioneira and Tamboara did not show satisfactory development in $C9^7$ and $C10^8$ culture media since the plantlets turned yellow, calluses were formed and shoots did not grow. GA_3 had to be added in C7 and C8 culture media for the efficient development of the meristems. It was also efficient to the growth of the Fécula Branca, IPR-União and Olho Junto seedlings in $C9^7$ and $C10^8$ culture media since the concentration of GA_3 (0.05 mg · L^{-1}) was maintained in the two culture media $C9^7$ and $C10^8$ (Figure 3).

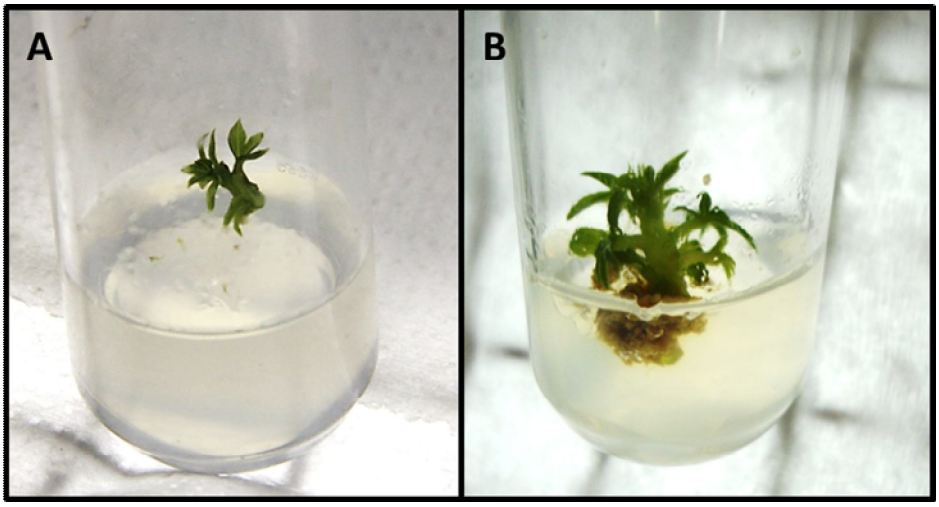

Figure 3 - Meristem tip development of the cassava cultivar IPR-União. **A:** plantlet generated in C7 culture medium after 60 days. **B:** Plantlet transferred to $C9^7$ culture medium after 30 days growth.

GA_3 had an effective role in the organogenesis of the cassava meristem tip development. Gibberellins´ main effect is the stimulation of the growth of organs

already formed. Oliveira et al. (2000) and Souza (2008) also used 0.05 mg · L^{-1} of GA_3 to obtain a good development of roots and aerial part of cassava plantlets.

The culture media C9[7] and C10[8] with their higher concentration of auxin and cytokinin than the culture media C7 and C8 provided a fast development, shoot elongation and root formation (Figure 4). Rapid root induction and elongation is one of the most important steps toward fast and successful production of *in vitro* regenerated shoots. The presence of roots in the cassava seedlings, proportional to the development of the aerial parts, is beneficial to multiplication; they provide the absorption of nutrient and the production of shoots which will be used as explants in the subcultures (Oliveira et al. 2000).

Figure 4 - Growth of cassava plantlets in culture media C9[7] and C10[8] provided A: shoot elongation and B: root formation.

Results of current experiments with cassava cultivars corroborate the literature that an elaborate and efficient regeneration system is essential for the development of several processes and the propagation of new cultivars (Raemakers, 2001). According to Smith et al. (2009), the composition and concentration of plant growth regulators in the culture medium are factors that determine growth and development of most tissues in the *in vitro* culture systems.

The *in vitro* regeneration of the cassava cultivars showed that meristem tip regeneration in cassava greatly depended on the genotype, and culture media were

effective in the regeneration capacity. The adaptation and optimization of culture conditions for the genotypes Baianinha, Icaraíma, Pioneira and Tamboara are still necessary. The levels and the interaction between the endogenous and exogenous hormones may cause difference in the regeneration ability of explants (Tang, 2008). Feitosa et al. (2007) have shown different results when evaluating the frequency of somatic embryogenesis and number of embryos per explant in two cassava varieties cultivated in the same culture medium. Oliveira et al. (2000) also pointed out that each tested genotype had a significantly different effect during *in vitro* development.

After the establishment of the cassava development protocol, two plantlets of Fécula Branca (FB1 and FB2) obtained from $C10^8$ culture medium, one plantlet of IPR União from $C9^7$, one plantlet of Olho Junto (OJ1) from $C9^7$ and another plantlet of Olho Junto (OJ2) from $C10^8$ were chosen and subcultivated (cut perpendicular to the midrib) into three sections: lower, middle and upper, with a shoot in each explant. The side shoots were also cut and each section was considered an explant. The plantlets were subcultured five times at every 40 days in their respective $C9^7$ and $C10^8$ culture media. After the five subcultures, 15 cloned plants were obtained from each FB1, FB2, IPRU, OJ1, and OJ2 plantlets.

Generated cloned plants were transplanted to plastic cups with substrate, covered by cups to maintain humidity for 1 week; they were then taken to the greenhouse and the cup was gradually removed (Figure 6). The regeneration capacity to survive under field conditions is important as it determines the success of *in vitro* propagation. The regenerated plants were successfully acclimated with a high survival rate (Table 3).

Table 3 - Acclimatization of cassava plants generated from *in vitro* culture

	Acclimatized plants	Survived plants	Survival rate
Fécula Branca 1*	15	15	100%
Fécula Branca 2*	15	14	93.3%
Olho Junto 1*	15	13	86.6%
Olho Junto 2**	15	14	93.3%
IPR – União**	15	13	86.6%

*Plantlets cultivated in culture medium with IBA. **Plantlets cultivated on culture medium with NAA.

Rooted plants were acclimatized successfully in the greenhouse, with a high survival rate and no observable morphological aberrations. The development of each meristem of the Fécula Branca variety in C8/C108 culture media generated 14-15 acclimated cassava plants after five subcultures in C108 culture medium.

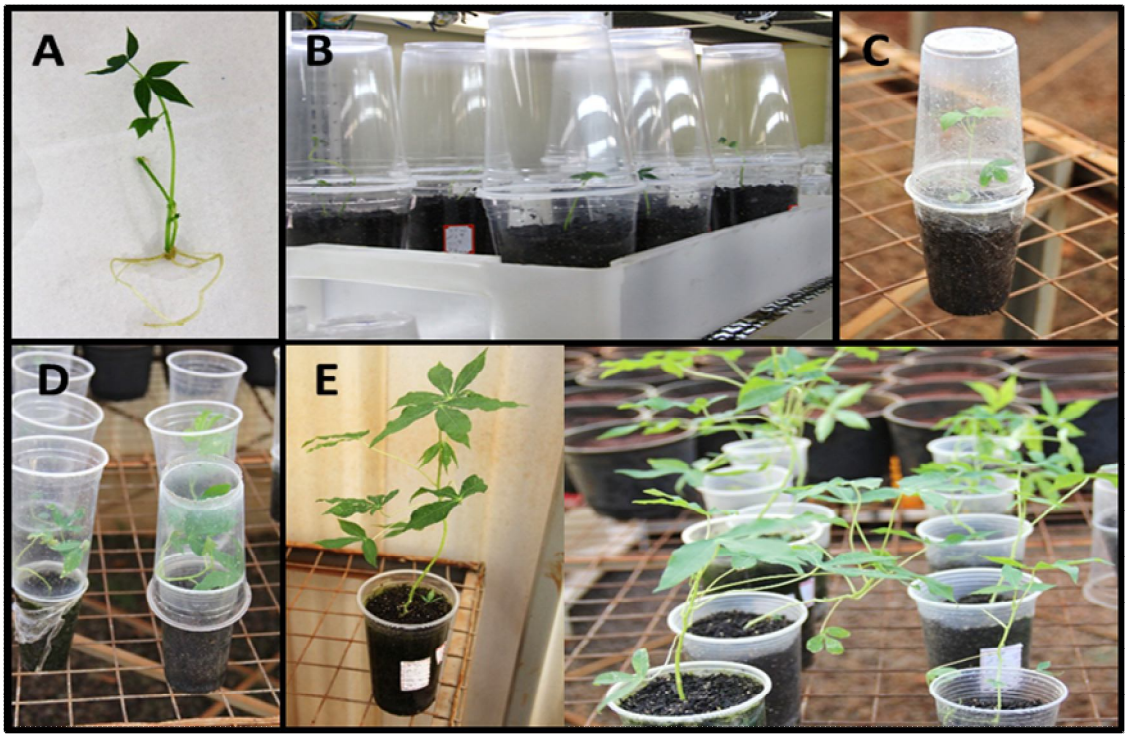

Figure 6 - **A:** Clean plantlet ready for acclimatization. **B:** Plantlets covered by plastic cup. **C:** Plantlets in the greenhouse. **D:** Plastic cup was removed gradually. **E:** successfully acclimated plant in the greenhouse.

Further, the development of each meristem of the IPR-União variety in C7/C9^{7} culture media generated 13 acclimated cassava plants after five subcultures in C9^{7} culture medium. Perspectives for the Fécula Branca variety propagated in the C8/C10^{8} culture media established in current study comprised the generation of 140-150 clones from one plant with 10 apical meristems, while the perspective for the IPR-União variety propagated in C7/C9^{7} culture media is the generation of approximately 130 clones. Similar reasoning and perspectives may be applied to the Olho Junto variety. Thus, it is possible to establish the clonal propagation of the Fecula Branca, IPR-União and Olho Junto varieties at a less than 12-month period and at a more than 10-fold proportion by employing the culture media developed in current study.

3.2. Virus indexing by PTA-ELISA and IC-RT-PCR

Leaves of 10 randomly selected plants were used for the PTA-ELISA assay after successful acclimatization. The cassava meristem-tips were inoculated with <0.5mm and PTA-ELISA index showed a low index virus-free regenerated plants (Figure 7).

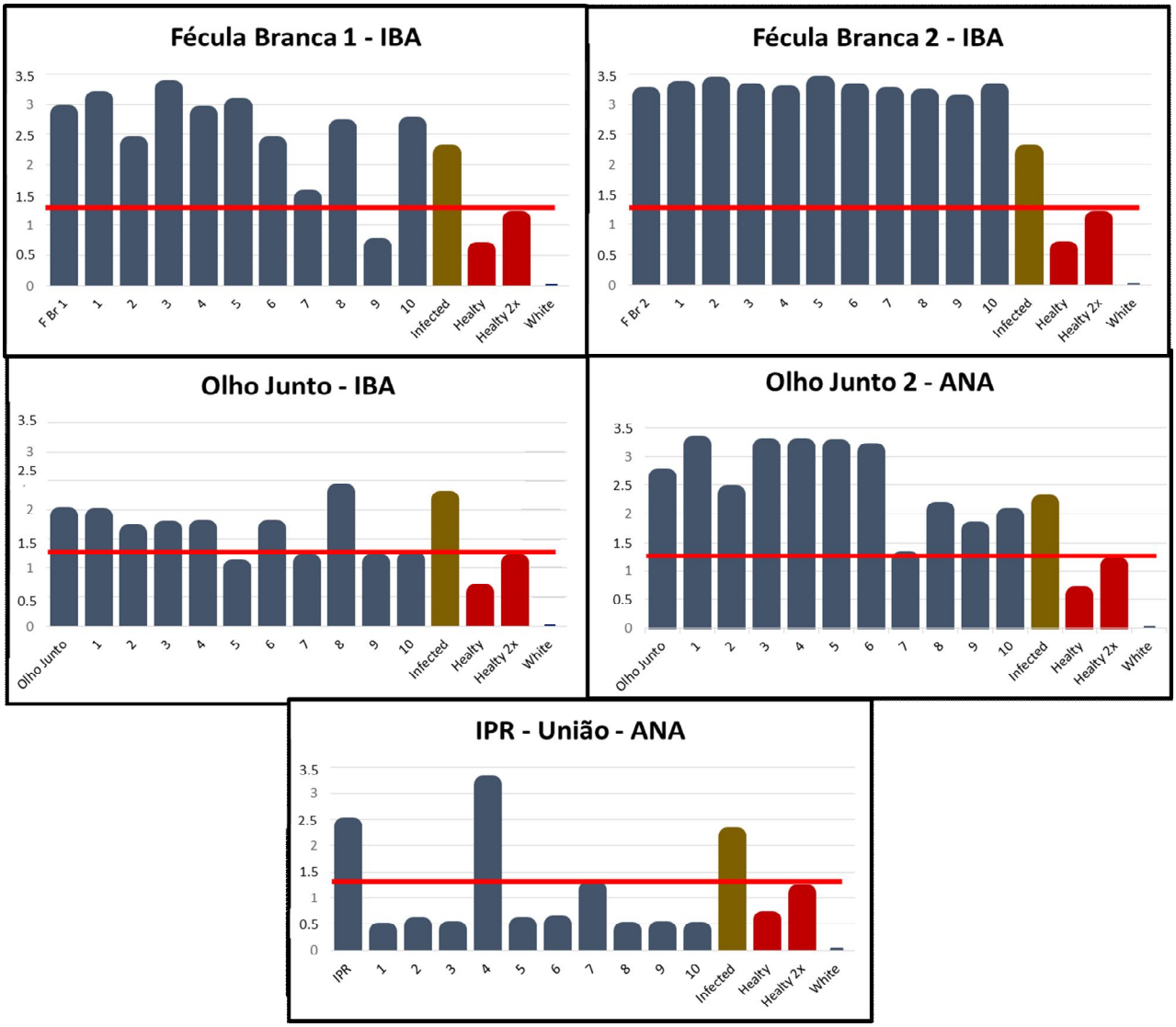

Figure 7 - PTA-Elisa assay of the acclimatized cassava plants. Each graph represents the mother plant and 10 cloned plants (1-10). The red line represents the minimum value to be considered virus free.

Only one of the plantlets was virus free in the Fécula Branca 1 samples. The Fécula Branca 2 did not have any virus-free plant. Whereas the Olho Junto 1 samples showed four virus-free plants by the PTA-ELISA assay, the Olho Junto 2

had only one virus-free plantlet. The IPR–União samples had the highest rate of clonal cleaning, with nine virus-free plants by PTA-ELISA indexing. Since the PTA-ELISA was not efficient to detect low virus levels, the samples were also analyzed according to IC-RT-PCR protocol developed by Silva et al. (2011) to increase the sensitiveness in the CsCMV virus detection (Table 4).

Table 4 - Virus-free plants of the cassava cultivars

Cultivar*	PTA-ELISA	IC-RT-PCR
Fécula Branca 1 (IBA)	10%	10%
Fécula Branca 2 (IBA)	0%	0%
Olho Junto 1 (IBA)	40%	20%
Olho Junto 2 (IBA)	10%	10%
IPR União (NAA)	90%	70%

*Exclusive analysis of plants generated by in vitro tissue culture.

IC-RT-PCR revealed that some plants identified as virus-free by ELISA were not clean. Although four plants in the Olho Junto 1 cultivar were virus-free by PTA-ELISA, two were indexed as infected by IC-RT-PCR. Two plants in the IPR-União cultivar were also false negative and demonstrated that IC-RT-PCR indexing was more sensitive than PTA-ELISA.

Results showed that most plants generated by *in vitro* culture have CMV virus infection. The technique was efficient for viral cleaning only in the case of the cassava cultivar IPR-União. In the IPR-União cultivar, 70% of the meristem-tip cultures were successful cloned and generated virus-free plants indexed by PTA-ELISA and IC-RT-PCR methods. The greatest problem in virus cleaning is that if the meristem tip is very small, the chance to recover a virus-free plant is very high, but the survival rate is low (Grattapaglia & Machado, 1990). The viral cleaning in meristem-tips of cassava also seems to be related with the level of viral infection in the parental plants used in obtaining the explants. This is due to the fact that a lower level of viral infection was detected in the parental IPR-União than that of levels of viral infection in parental plants of the Fécula Branca and Olho Junto varieties prior to *in vitro* propagation (Figure 1). Although the parental plants of the Icaraima cultivar showed no previous viral infection, their in vitro development was not satisfactory since these plants did not grow and did not produce shoots in the subculture.

3.3. Genetic relationship among the parental plants of the three cassava cultivars propagated in vitro

The primers combinations (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5) amplified a total of 72 DNA segments of which 43 (59.72%) were polymorphic in parental plants of the three cassava cultivars Fécula Branca (FB1, FB2), IPR-União (IPRU), and Olho Junto (OJ1 and OJ2) propagated *in vitro* (Table 4). The size of amplified products ranged between 115 pb in the AYF5xAYF9 primer and 2.5kb in the AYF2xAYF3 primer. The primer AYF2xAYF3 amplified the highest number (16) of DNA segments with 37% of polymorphism. The highest polymorphism level (76.92%) was shown by the primer AYF9. The IRAP and REMAP markers system have been used for studying the genetic diversity and structure genetics of populations in helianthus (Vukich et al. 2009), flax (Smýkal, et al. 2011) and maize (Kuhn et al. 2014), while a high genetic diversity was observed in the cassava varieties analyzed in current study.

Table 4 - Polymorphism of markers IRAP and REMAP by the analysis of the cassava cultivars Fécula Branca 1, Fécula Branca 2, IPR-União, Olho Junto1 and Olho Junto2

Primer	TNS	PS	%PS
AYF8	11	8	72.72%
AYF2 x AYF3	16	11	68.75%
AYF5 x AYF9	11	4	36.36%
AYF3 x AYF8	12	6	50.00%
AYF4 x SSR5	9	4	44.44%
AYF9	13	10	76.92%
Total	**72**	**43**	**59.72%**

TNS: Total number of amplified segments; PS: polymorphic DNA segments

The dendrogram by Jaccard coefficient (Figure 5) showed that the molecular marker IRAP and REMAP analysis divided the parental cassava plants into two groups (Figure 5). The first group was formed by the Fécula Branca 1 (FB1), Fécula Branca 2 (FB2) and IPR-União (IPRU) plants, while the second group was formed by the Olho Junto 1 (OJ1) and Olho Junto 2 (OJ2) plants. The highest similarity

coefficient (0.8769) was observed between FB1 and FB2 plants which generated cassava plantlets in culture medium supplemented with IBA. The lowest similarity coefficient (0.6176) was observed between OJ2 and FB1 plants which were in separated groups and generated plantlets in culture medium containing different auxin types.

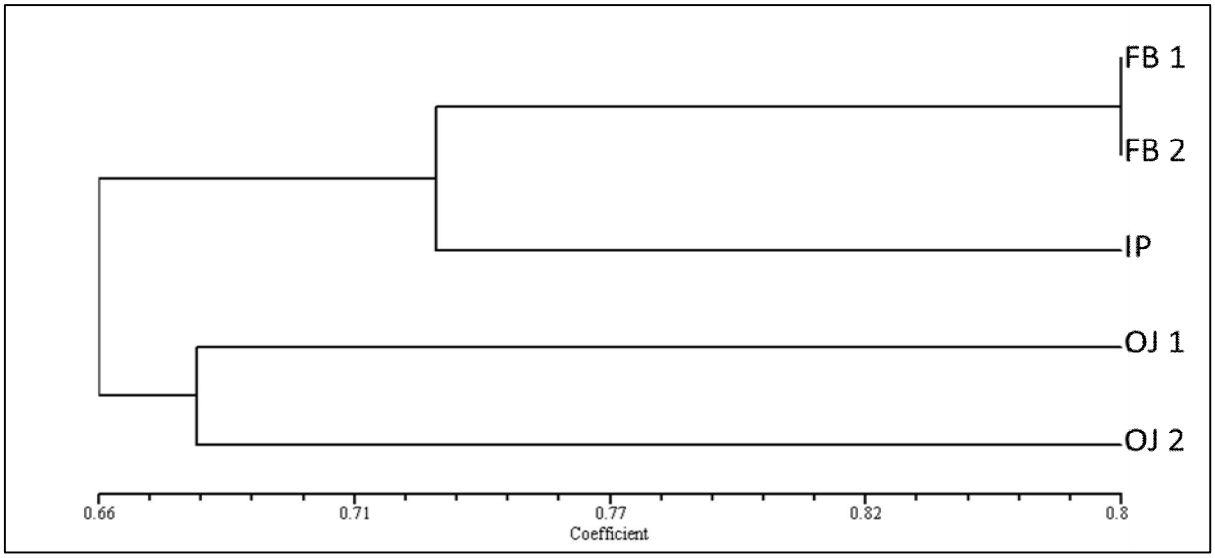

Figure 5 - Dendrogram from the parental plants, using molecular marker based on retrotransposon (IRAP and REMAP). FB 1 (Fécula Branca 1), FB 2 (Fécula Branca 2), IP (IPR-União), OJ 1 (Olho Junto 1), OJ 2 (Olho Junto 2).

A low similarity coefficient (0.6799) was also observed between OJ1 and OJ2 plants which generated plantlets in culture medium respectively containing IBA and NAA.

Table 6 - Matrix of genetic similarity showing the relationship between cassava varieties used for meristem propagation

	FB 1	FB 2	IPRU	OJ 1	OJ 2
FB 1	1.0000				
FB 2	0.8769	1.0000			
IPRU	0.7164	0.7424	1.0000		
OJ 1	0.6567	0.6567	0.6507	1.0000	
OJ 2	0.6176	0.6417	0.7166	0.6779	1.0000

FB 1 (Fécula Branca 1), FB 2 (Fécula Branca 2), IP (IPR-União), OJ 1 (Olho Junto 1), OJ 2 (Olho Junto 2).

The similarity coefficient (0.7166) between IPRU and OJ2 plants which generated plantlets in culture medium containing NAA was higher than the similarity between OJ1 and OJ2 (0.6779). However, the similarity coefficient in OJ1 and FB1 and FB2 which generated plantlets in IBA-supplemented medium was also low (0.6567) and indicated that high or low similarity coefficient may not be used to monitor the selection of the culture medium supplemented with the same or with different types and concentrations of growth regulators. Our forecast that the meristem-tip culture of cassava cultivars showing high genetic similarity may be established in culture medium with the same supplements was successful. On the other hand, different genotypes (OJ1 and FB1) showing low genetic similarity (0.6567) were also established in culture medium supplement with the same types and concentrations of growth regulators. Our results foreground the premise that the tissue culture is an empirical science and difficult to predict; it is necessary to set appropriate conditions for the in vitro cultivation of different cassava genotypes. However, the preliminary suggestion is that for genotypes with the highest genetic diversity (low genetic similarity; for example, the Olho Junto cassava cultivar) may be micro-propagated with different growth regulators as supplements in the culture medium. It is a suggestion that requires further investigation. The genetic variability within a variety may determine their development in the culture medium with one or more growth regulators. In fact, when an explant is inoculated into a culture medium, the responses to exogenous growth regulators depend to a great extent on the physiological status of the donor plant, which depends on the information codified at the DNA level.

4. CONCLUSIONS

a) *In vitro* propagation of cassava (*Manihot esculenta*) from apical meristems is a fast procedure but requires elaborate intermediary strategies with changes in the growth regulators concentrations;

b) Besides the different types and concentrations of auxin (IBA and NAA) and cytokinin 6BA, the GA_3 (0.05 mg $\cdot$ L^{-1}) is indispensable for the apical meristems propagation of different cassava varieties;

c) *In vitro* propagation of cassava (*Manihot esculenta*) is dependent on the genotype and also on the genetic diversity within each variety.

d) The similar genotypes can be cultivated on the same auxin, however this depend of the physiological characteristic of the variety.

e) The indexing by IC-RT-PCR is more sensitive than PTA-ELISA.

f) The low rate of virus-free plants obtained suggests that the technique needs to be improved, the small meristem don't survive however the big meristem keeping infected by the virus. Anyway, the 10% of efficiency is enough to provide explant virus-free.

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CAPÍTULO II

DEVELOPMENT OF RETROTRANSPOSON-BASED MARKERS IRAP AND REMAP FOR CASSAVA (*MANIHOT ESCULENTA*) GENOME

ABSTRACT

Retrotransposons are abundant in the plant´s genome. Inter-retrotransposon amplified polymorphism (IRAP) and the retrotransposon-microsatellite amplified polymorphism (REMAP) markers were developed for the cassava genome (*Manihot esculenta* Crantz) in current study. Four cassava cultivars (Fécula Branca, IPR-União, Olho Junto and Tamboara, two samples per cultivar) were used to obtain IRAP and REMAP fingerprints. Twelve primers designed were amplified alone and in combinations. The 42 IRAP/REMAP primer combinations amplified 431 DNA segments (bands; markers) of which 36 (8.36%) were polymorphic. The largest number of informative markers (16) was detected using the primers AYF2 and AYF2xAYF4. The number of bands for each primer varied from 3 to 16, with an average of 10.26 amplified segments per primer. The size of amplified products ranged between 100 and 7,000 bp. The AYF2 primer generated the highest number of amplified segments and also showed the greatest number of polymorphic bands (68.75%). Two samples of each cultivar of cassava were used to illustrate the usefulness and the polymorphism of IRAP/REMAP markers. IRAP and REMAP markers producing high number of reproducible bands, and might be even more informative and reliable for the study of genetic diversity and relationship among cassava cultivars.

Keywords: retrotransposons, cassava, diversity, primer design.

1. INTRODUCTION

The cassava (*Manihot esculenta* Crantz), native to South America, was domesticated about 8000 years ago (Bull et al., 2011). The tuberous roots are an important carbohydrate source due to their high starch contents (Meji-Atuero et al., 2012). Cassava is one of the most important crops worldwide since it is the predominant food in the staple diet of several countries in Africa, Asia and Latin America (McMahon et al., 1995). It is also used for animal feed and as raw material for starch production (Wongtiem et al., 2011). The cassava is also important for activities not associated with food, such as for textile and paper industries (Meji-Atuero et al., 2012) and for ethanol production, used as biofuel (Wongtiem et al., 2011). Thus, knowledge on the genetic diversity and relationships among cassava cultivars is an important requisite to improve and increase the production of different cultivars.

Since there are few reliable phenotypic morphological characteristics in the *Manihot* genus (Olsen and Schaal, 2001), molecular markers have been used to infer the genetic relationships among cassava cultivars. Several molecular markers, such as isozymes (Rezende et al., 1999), randomly amplified polymorphic DNA (RAPD) (Zacarias et al., 2004), amplified fragment length polymorphisms (AFLP) (Aguirre et al., 1999), simple sequence repeats (SSR) (Aguirre et al., 1999), (Siqueira et al., 2009, Lekha et al., 2010), and simple sequence repeats from the expressed sequence tags (EST-SSR) (Kunkaew et al., 2011, Sraphet et al., 2011) markers have been used to analyze the genetic diversity and relationship among cassava cultivars. However, the retrotransposon markers, a highly promising feature for phylogenetic studies, gene mapping, and genetic diversity (Kalendar et al., 1999), have not yet been analyzed in cassava cultivars.

Retrotransopons are the most abundant transposable elements in the eukaryote genomes and are classified into LTR (Long Terminal Repeat) and non-LTR (Kumar and Bennetzen, 1999). Retrotransposons are dispersed in the plant´s genomes and some retrotransposon families are represented by thousands of copies (Kalendar et al., 2011). The new copies of retrotransposons are inserted randomly in preexisting sequences of the genome by copy-paste system and, consequently, increase the copy numbers (Kalendar and Shulman 2006). The retrotransposons

contribute to size, structure, variation and diversity of the genome; they have a great effect in the *gene* function and represent a high percentage in genome contents (Gbadegesin and Beeching 2010).

The S-SAP (sequence-specific amplification polymorphism) was the first molecular marker based on retrotransposon to be developed. It is based on a primer annealing in the end of the retrotransposon and a site corresponding to an AFLP restriction enzyme (Waugh et al., 1997). Two other systems of retrotransposon-based molecular marker, IRAP and REMAP, were also developed by Kalendar et al., (Kalendar et al., 1999). The IRAP (Inter-Retrotransposon Amplified Polymorphism) consists in the amplification by PCR (Polymerase Chain Reaction) of DNA sequences between two nearby retrotransposons, using a primer designed from the LTR sequence of a retrotransposon. On the other hand, REMAP (Retrotransposon-Microsatellite Amplified Polymorphism) uses a primer based on the LTR sequence and also a SSR primer with an anchored nucleotide (non-SSR) at the 3' end of the primer.

One hundred and fifty-four transposable elements of the cassava genome were partially sequenced by Gbadegesin et al. (2008): 59 families of *Ty1/copia*, 26 families of *Ty3/gypsy* retrotransposons, and 40 families of *En/Spm* transposons. The cassava genome has been sequenced but no primers have been designed for the IRAP and REMAP markers to study the specie. In current study, molecular markers IRAP and REMAP, specific for the cassava genome, have been developed.

2. MATERIAL AND METHODS

2.1 Plant material and DNA isolation

The cassava varieties “Fécula Branca” and “Olho Junto” (with low cyanogenic acid content), “IPR-União” and “Tamboara” (with high cyanogenic acid content) were used in the present study (Table 1). The four cassava cultivars, the most cultivated in the state of Paraná, Brazil, were obtained from the IAPAR (Agronomic Institute of Paraná – Brazil) germplasm bank.

Table 1 - Cassava cultivars, content of cyanogenic acid and utility of the four cultivars (IAPAR)

Cultivar	Cyanogenic acid	Utility
Fécula Branca	Low	Immediate human consumption
IPR-União	High	Industry
Olho Junto	High	Immediate human consumption
Tamboara	High	Industry

Total DNA from each cultivar was isolated using CTAB (cetyltrimethylammonium bromide) protocol Knapp and Chandlee (1996), adapted by Carvalho (Cenargen – Embrapa). The genomic DNA was dissolved with TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and quantified with picodrop (Pico200 spectrophotometer).

2.2. Primer design

The primers for the LTR sequences of the IRAP and REMAP markers were designed from cassava sequence database, while primers for the SSR sequences of the REMAP markers were randomly designed and further tested to validate their utility. Retrotransposon sequences from three families of transposable elements in the cassava genome were described Gbadegesin et al. (2008) and deposited in the GeneBank as AY946045–AY946199 accessions. The retrotransposon sequences obtained from GeneBank were submitted to Phytozome Blast, to find the adjacent sequences of the retrotransposon. The adjacent sequences of the retrotransposon

were then analyzed by the LTR finder program for the LTR sequences. Twelve different LTR sequences were obtained and aligned in the CLUSTAL W software (Thompson et al., 1994). The primers, based on the most conserved region for each LTR with amplification directed outwards of the retrotransposon, were designed (Kalendar and Shulman, 2006). One or two LTR primers were used in the same reaction for the IRAP markers (Figure 1).

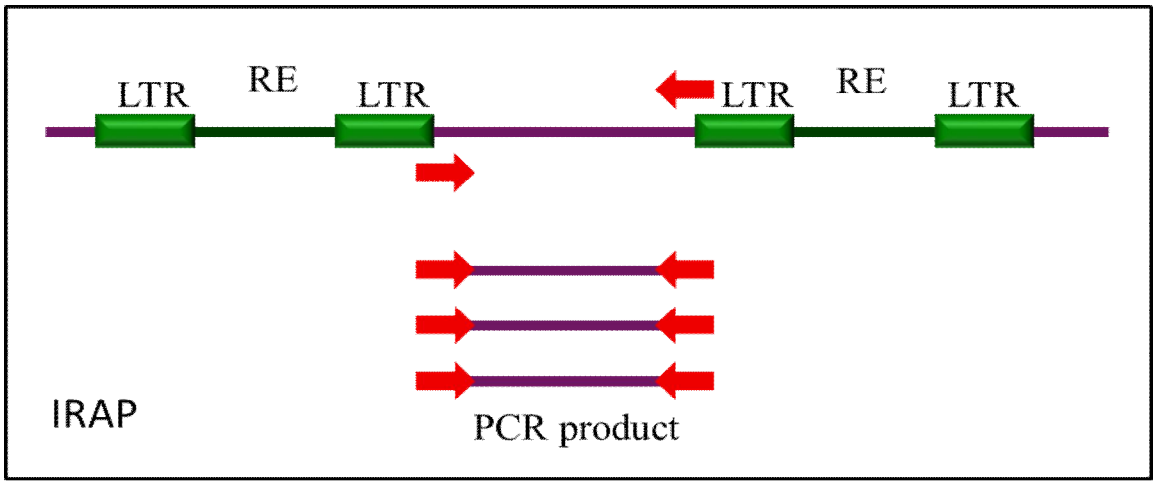

Figure 1 - Product from IRAP amplification. IRAP: The primer amplified the sequence between two retrotransposons. LTR: Long Terminal Repeat; RE: Retrotransposon. Modified from Kalendar and Shulman (2006).

One LTR primer and one SSR primer (with two or three repeated selective bases) were used for the REMAP markers (Figure 2).

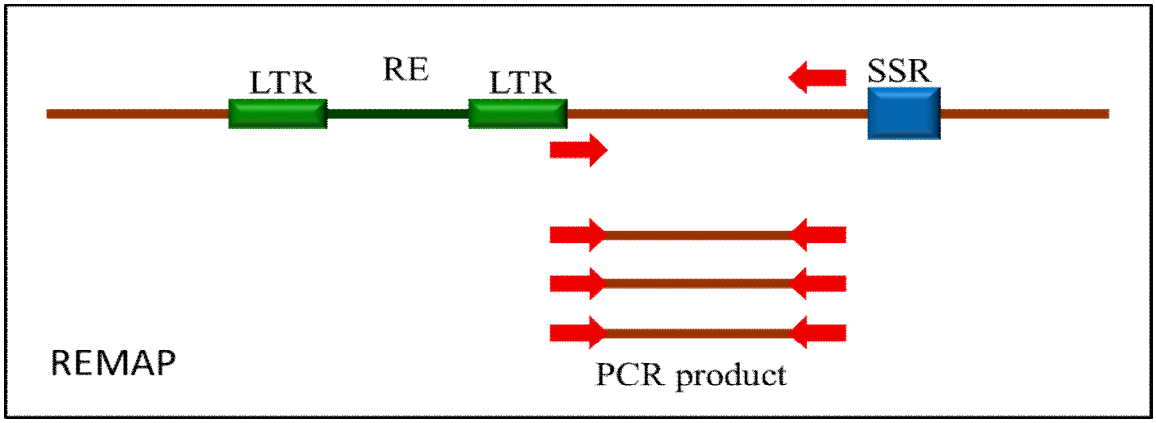

Figure 2 - Product from REMAP amplification. REMAP: The primer amplified the sequence between a retrotransposon and a SSR sequence. LTR: Long Terminal Repeat; RE: Retrotransposon; SSR: Simple Sequence Repeat. Modified from Kalendar and Shulman (2006).

2.3. IRAP and REMAP amplification

PCR reactions were performed in 25 µL volume: 30 ng of DNA, 1xPCR buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 4 pmol of each primer, 100 µL dNTP, and 1 U of PrimeSTAR DNA polymerase (Takara Bio, Tokyo, Japan). Amplification was performed in a MJ Research PTC-200 PCR Peltier Thermal Cycler (Bio-Rad, Hercules, USA). After 10 min of initial denaturation at 95 °C, amplifications were carried out in 36 cycles at 94 °C for 30 sec; at 55 °C for 30 sec and at 72 °C for 3 min, with a final extension of 10 min at 72 °C. The PCR products were identify by electrophoresis with 3% agarose (ECOGEN, AG-0600, Madrid, Spain) in 1xTAE buffer followed by EtBr staining and UV visualization. PCRs were repeated three times with similar results. Each primer was tested for efficiency in the yield of IRAP segments and for fingerprint quality (separate and well definite bands in the gel).

2.4. Data Scoring and Analysis

IRAP and REMAP bands were interpreted as 1 for presence or 0 for absence. Binary matrices (presence/absence) were prepared from IRAP and REMAP products. Each PCR product represented a single locus. Monomorphic bands were removed from the raw scored data sets. The weakly stained bands were not scored. The Dice coefficient was employed to estimate genetic similarity. The binary data obtained were then analyzed with NTSYSpc 2.1 program (Rohlf, 1989), which generated the genetic distance matrix (Nei and Li, 1979) and drew the UPGMA dendrogram.

3. RESULTS AND DISCUSSION

3.1. Development of IRAP for cassava germplasm genotyping

Twelve LTR retrotransposon families were identified from the cassava partial sequences deposited in databases using the LTR finder. Several LTR sequences were obtained for each family and aligned with ClustalW (Thompson et al., 1994). The most conserved regions were used to design primers, with 21-24 bp and high C:G proportion. Primers were located next to the end of the retrotransposon element facing outwards of the LTR (Table 2). Twelve retrotransposon primers were designed and all primers were used for the analysis, alone or in combinations.

Table 2 - Selected primers used to generate IRAP and REMAP in cassava

Name	Class	Primer	%GC
AYF1	LTR	3' GGATCCTAGCGCCGGTAGCGGT 5'	68.18
AYF2	LTR	3' CTCTCTGTACGCTCCTGTTCGTAC 5'	54.17
AYF3	LTR	3' ACGGCTTCGACAACCGCTTATC 5'	54.55
AYF4	LTR	3' GACCGGCTTCGACAACCGCGAGTC 5'	66.67
AYF5	LTR	3' CCTAGCGCCGGTAGCGGTCCGA 5'	72.72
AYF6	LTR	3' CCGGAGTCTAGCCGGGTATTAC 5'	59.09
AYF7	LTR	3' GAACAAGTTATGCTATTATGCA 5'	31.81
AYF8	LTR	3' CCAAGGCTAGTTGCTCCATGTC 5'	54.54
AYF9	LTR	3' AGTCTCTAATAGGCTGGGGTTC 5'	50.00
AYF10	LTR	3' CAAATATCTTATGTGATAGAG 5'	28.57
AYF11	LTR	3' CATTGAAGATATAACTTATCCT 5'	27.27
AYF12	LTR	3' CTTCTCTCTCTGTACGCTCCTG 5'	54.54

Each LTR primer was tested alone and with all possible combinations for IRAP markers. In the case of REMAP, four LTR primers were combined with four SSR primers. The scoring criteria were the number, intensity and sharpness of the PCR products, as well as the degree of polymorphism among the *genotypes*. Seventy-eight combinations of the LTR primers were used in one cassava sample and 53 sharpness bands were observed in the gel (49 IRAP and 4 REMAP) so that

applicability of the selected markers could be explored. The patterns of amplification of the different primer combinations were initially analyzed using genomic DNA extracted from two samples of each cassava cultivar and all primers´ combinations which produced bands were used to amplify the DNA from the four cassava cultivars (Fécula Branca, Olho Junto, IPR-União, and Tamboara). All the combinations of primers tested that yielded either poor amplifications or few products were discarded from the analysis. The 42 primers combinations used in the analysis were 40 IRAP and 2 REMAP (Table 3).

Table 3 - Combinations of primers used to generate IRAP and REMAP markers in cassava showing the total of amplified segments (TAS) as well as the number of polymorphic amplified segments (PAS)

Primer(s)	TAS	PAS	Primer(s)	TAS	PAS
AYF1	10		AYF2xAYF8	14	
AYF2	16	11	AYF2xAYF9	13	1
AYF4	15	1	AYF3xAYF4	7	
AYF5	9		AYF3xAYF5	5	
AYF8	15	4	AYF3xAYF8	13	3
AYF9	10	2	AYF3xAYF9	15	1
AYF12	12		AYF4xAYF5	9	
AYF1xAYF2	13	1	AYF4xAYF7	14	
AYF1xAYF3	12	4	AYF4xAYF8	13	
AYF1xAYF4	7		AYF4xAYF9	14	
AYF1xAYF5	12	1	AYF4xAYF11	9	
AYF1xAYF6	3		AYF4xAYF12	7	
AYF1xAYF8	10	2	AYF5xAYF6	4	
AYF1xAYF9	10		AYF5xAYF8	9	
AYF1xAYF10	9		AYF5xAYF9	11	1
AYF1xAYF11	9		AYF5xAYF10	10	1
AYF1xAYF12	11		AYF5xAYF11	9	1
AYF2xAYF3	12	2	AYF8xAYF9	12	
AYF2xAYF4	16		AYF9xAYF12	8	
AYF2xAYF5	7		AG8 xAYF1	8	
AYF2xAYF6	3		AC8 xAYF5	6	
			Total	**431**	**36**

The 42 IRAP/REMAP primer combinations amplified 431 DNA segments (bands; markers) of which 36 (8.36%) were polymorphic (Table 3). The total number of bands for each primer varied between 3 and 16, with an average of 10.26

amplified segments per primer. The size of amplified products ranged between 100 and 7,000 bp. Primers AYF2 and AYF2xAYF4 produced the largest number of bands (16 bands). Fifteen out of the 42 primer combinations tested produced polymorphic bands and thus were useful for investigations of genetic variability in the cassava genome and to establish the genetic relationship among cassava cultivars. The largest number of informative markers was detected using the primer AYF2 (11 bands, 68.75% of the total amplified), while the primer combinations AYF8 and AYF1xAYF3 produced 4 polymorphic bands (Figure 3).

Figure 3 shows that the primer AYF2 is also used to characterize the cassava´s intra cultivar polymorphism. The evident polymorphism in only two samples of the IPR União cultivar (Figure 3) indicated that the IRAP/REMAP markers may also be useful to investigate the genetic variability within cassava cultivars. Primers AYF4, AYF8, AYF3xAYF8 and AYF5xAYF10 also showed polymorphism within Tamboara and Fécula Branca cassava cultivars (results not shown).

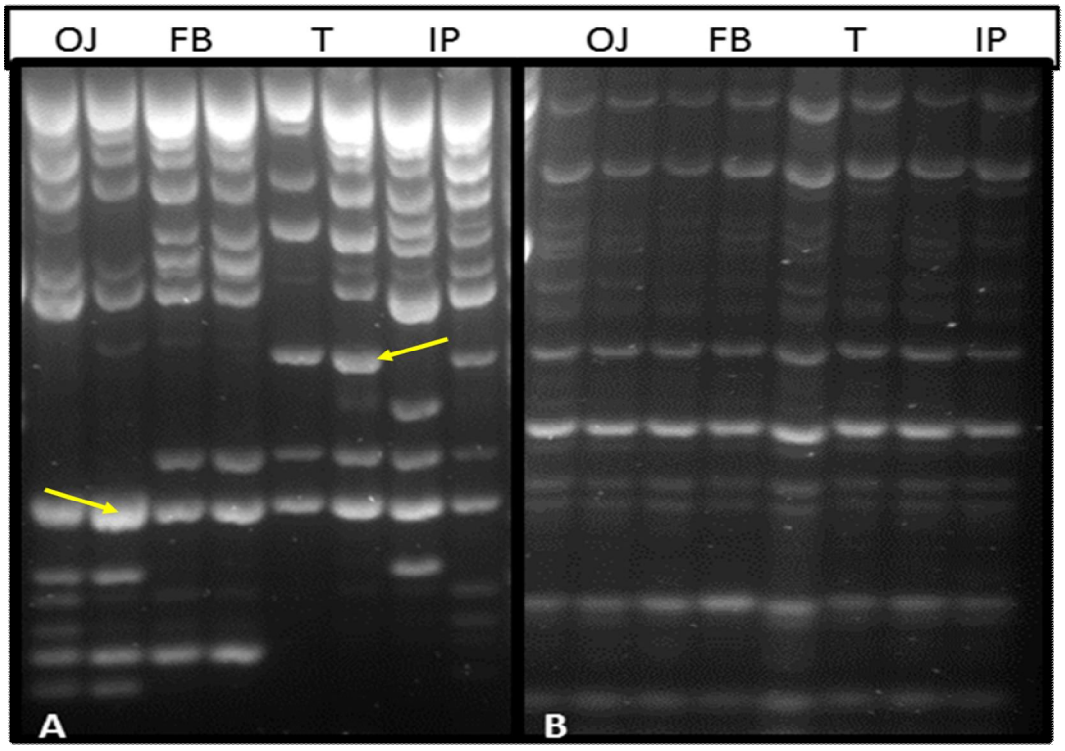

Figure 3 - IRAP gels from the four cassava cultivars, showing the polymorphism. A: primer AYF2 (11 polymorphic bands); **B**: AYF8 (4 polymorphic bands). Samples: OJ = Olho Junto, FB = Fécula Branca, T = Tamboara, IP = IPR-União. Arrows in the left indicate monomorphic amplified band, arrows in the right indicate polymorphic amplified band.

3.2. Genetic Diversity among cassava cultivars using IRAP/REMAP makers

The percentage of polymorphic segments ranged from 1.21% in the IPR União and Fécula Branca samples (2 IRAP/REMAP markers) to 4.85% in the Tamboara sample (8 IRAP/REMAP markers). In Olho Junto samples, the percentage of polymorphic segments was null (polymorphism was absent) and the genetic distance among the four samples was 0.078. The UPGMA dendrogram obtained from the cluster analysis of Nei's unbiased genetic distance [25] revealed one main group comprising the cassava cultivars IPR União, Fécula Branca, and Tamboara, while the samples of the Olho Junto formed an isolated group (Figure 4). The values of Nei's identity (I) varied from 0.8567 (between the Olho Junto and Tamboara samples) to 0.9431 (between Fécula Branca and Tamboara samples) (Table 4). According to these results IPR União, Fécula Branca and Tamboara are grouped together and Olho Junto lies in a different group (Figure 4).

Table 4 - Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Variety	Olho Junto	Fécula Branca	Tamboara	IPR União
Olho Junto	****	0.8906	0.8567	0.8730
Fécula Branca	0.1158	****	0.9431	0.9340
Tamboara	0.1547	0.0586	****	0.9355
IPR União	0.1359	0.0683	0.0667	****

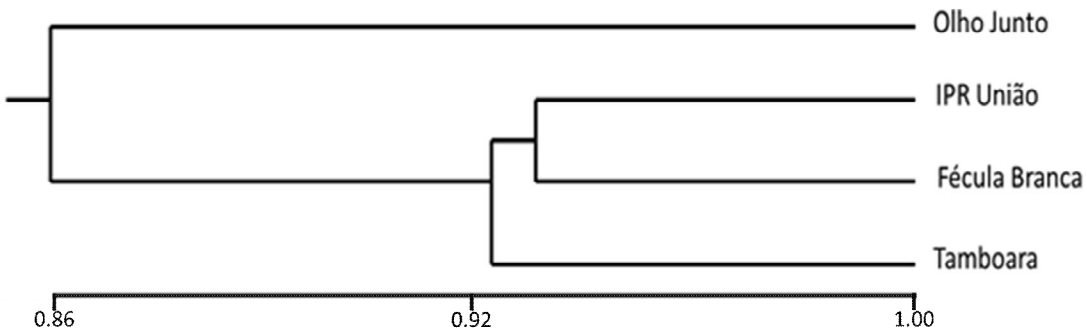

Figure 4 - Dendrogram from four cassava cultivars. An unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis based on genetic distances of Nei and Li (1979) obtained from the IRAP and REMAP primers, among cassava cultivars.

Cassava genome was estimated at about 772 Mpb. 59 families of transposable elements were detected including *Ty1/copia* and 26 *Ty3/gypsy* retrotransposons and 40 *En/Spm* transposons (Gbadegesin et al., 2008). Further, the abundance of LTR retrotransposons in cassava genome was employed to develop primers for the IRAP and REMAP marker systems. The IRAP and REMAP markers systems were used to study the genetic diversity in *Heliantus* (Vukich et al., 2009), flax (Smýkal et al., 2011) and maize (Kuhn et al., 2014), among others. They were also applied to analyze the genetic diversity in cassava. Actually, in-depth knowledge on the genetic diversity in cassava cultivars is highly important for further use in breeding programs and conservation in germplasm banks.

The abundance of retrotransposons LTRs in cassava was confirmed in current study since 431 amplified DNA segments (molecular markers) were obtained with 42 IRAP and REMAP primer combinations. The AYF3, AYF6, AYF7, AYF10 and AYF11 primers failed to amplify any band when they were used alone, but they generated bands when used in combination with other primers (Table 3). The number of IRAP/REMAP markers obtained in current study (431) using only two samples of each Olho Junto, Fecula Branca, IPR União, and Tamboara cassava cultivar was higher than the number of the molecular markers used in several other studies of the cassava genome. Molecular markers, such as isozymes (22) (Aguirre et al, 1999); (82) (Resende et al., 2004), RAPD markers (311) (Zacarias et al., 2004), and SSR markers (43) (Aguirre et al, 1999); (46) (Siqueira et al, 2009), and EST-SSR markers (425) (kunkaew et al. 2011) produced the lowest number of reproducible bands. Although S-SAP is a co-dominant marker retrotransposon, the enzymatic steps may include the possibility of generating impurities and incorrect digestion (Kalendar and Shulman, 2006). When compared to other DNA markers, IRAP and REMAP markers are a simple technique, easy to operate, producing a high number of reproducible bands, and may be even more informative and reliable for the study of genetic diversity and relationship among cassava cultivars. Only two samples of each cultivar of cassava were used in current analysis just to illustrate the usefulness and polymorphism markers IRAP/REMAP.

Estimates of polymorphisms, genetic distance and genetic identity are illustrative and may not be used to provide conclusive information on the genetic diversity among the four cultivars analyzed. The number of cultivars (four) and samples analyzed of each cultivar was low (two samples of each cultivar) to group

them according to different contents of cyanogenic acid, for instance. However, a larger number of samples of each cultivar Olho Junto, Fécula Branca, IPR União, and Tamboara, as well as other cassava cultivars, will be analyzed using the IRAP/REMAP markers to investigate the genetic diversity and relationship among them, and even to detect correlations between phenotypic characters and retrotransposon markers. Knowledge on the genetic diversity and relationship among cassava cultivars is an important requisite to improve and increase production of different cultivars.

4. CONCLUSIONS

The IRAP and REMAP primers design for this study was effective to the assessment of cassava diversity. The retrotransposon-based marker was efficient to differentiate the four cassava cultivars and find the diversity within the samples. The application of these molecular marker can be a useful tool for breeding and analysis of diversity of cassava and another organisms.

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CAPÍTULO III

ANALYSIS OF SOMACLONAL VARIATION IN CASSAVA (*MANIHOT ESCULENTA*) BY MOLECULAR MARKERS

ABSTRACT

The cassava is propagated by meristem-tip culture, although no data are extant on the somaclonal variation in the species. Somaclonal variation is negative in germplasm maintenance, whereas it may result in loss in production and in commercial propagation. The marker EST-SSR and the retrotransposon-based markers IRAP and REMAP were used to detect somaclonal variations in cassava plants propagated *in vitro* at Stage 5 and Stage 10 of the subculture. Five groups of cloned plants were analyzed: Fécula Branca 1, Fécula Branca 2, IPR-União, Olho Junto 1 and Olho Junto 2. EST-SSR analysis revealed polymorphism in Fécula Branca 2, IPR-União and Olho Junto 2 cassava clones. Since IRAP and REMAP molecular markers produced 72 amplified bands, polymorphism was shown in all analyzed groups, indicating that IRAP and REMAP markers were more sensitive than the EST-SSR marker. The highest index of polymorphism was found in plants at Stage 10 and indicated that when more subcultivated plants are submitted, more somaclonal variations are generated.

Key words: Cassava, Somaclonal Variation, EST-SSR, IRAP and REMAP.

1. INTRODUCTION

Good Quality and fast propagation are the characteristics of *in vitro* plants. The conventional clonal propagation rate for the cassava is approximately ten plants between one and 12 months, and the ratio of *in vitro* culture is five plants between one and six weeks (Souza et al. 2008). However, plants in *in vitro* culture are under stressed conditions which may cause mutation and genetic changes in the cultured tissues, called somaclonal variation by Larkin and Scowcroft (1981). Since 1981, several papers have demonstrated and quantified morphological, cytological, and biochemical changes in somaclones or plants regenerated *in vitro*. High somaclonal variations at morphological, biochemistry and molecular levels have been reported in somaclones of *Cereus peruvianus* (Mangolin et al., 1997; Machado et al., 1999; 2000; Resende et al., 2007; 2010; Tavares et al., 2011). Santos (2004) reported somaclonal variations in different genotypes of banana propagated by the same protocol. The somaclonal variation may also be used as a tool to generate variability and to produce commercially viable plants (Araújo, 2004). However, somaclonal variation may not be desired when micropropagation aims at producing genetically uniform seedlings (Celedon, 2000). In fact, genetic uniformity is needed for the commercial propagation of some species (Bhojwani, 1996). The somaclonal variation generated by *in vitro* culture could result in liabilities to producers when seeking uniformity for large-scale production (Silva et al., 2010). In this case, somaclonal variation is a negative factor.

Among the various molecular markers available in the literature to study the genome of plants, the loci of simple repeated DNA sequences (SSR loci, also called microsatellite loci) have been identified as the most adequate due to the high polymorphism revealed by these markers. The above-mentioned technique is actually one of the best choices in the characterization of varieties and cultivars (Faleiro et al., 2001; Sereno et al., 2006; Lanaud et al., 1999). EST-SSR primers were designed by Raji et al. (2009b) based on EST (expressed sequences tags) sequences, identified by Lokko et al. (2007), to characterize the germplasms of cassava and their possible application in genetic improvement. The EST-SSR primers were tested in cassava cultivars from Africa, North America, South America and Asia. Altogether 124 new and unique EST-SSR primers were developed by Raji

et al. (2009a) and proved to be useful for the identification of proteins, mainly related to response to water stress (Lokko et al., 2007). EST collections were also generated by Lopez et al. (2004) from the analysis of mRNAs expressed in cassava starch and associated the sequences with susceptibility or resistance to the pathogen *Xanthomonas axonopodis*. Plants´ EST sequencing demonstrates that many proteins are encoded by gene families (quantitative characteristics). Raji et al. (2009a) and Lopez et al. (2004) identified EST-SSR sequences in cassava genome used by Kunkaew et al. (2011) to design EST-SSR primers.

Besides microsatellites, the plant retrotransposons may also be affected by *in vitro* tissue culture since biotic and abiotic stress affects their activity (Bradley, 2011). The transposons or transposable elements are DNA sequences that move from one position to another and constitute a significant portion of the genome (Snustad & Simmons, 2011). Du et al. (2009) showed that retrotransposon-based markers are an important tool in the study of genetic instability and genomic evolution. In a pioneer study on retrotransposons Hirochika (1993) discovered that they moved into the *Nicotiana tabacum* genome during the *in vitro* cultivation process. It was the first demonstration of retrotransposons activity of plants in tissue culture. Kalendar et al. (1999) developed two new methods for retrotransposon analysis: Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP). IRAP comprises the PCR amplification of DNA sequences between retrotransposons near copies, using a primer design based on Long Terminal Repeat (LTR) sequence end of a retrotransposon. A series of DNA segments whose size may be determined by electrophoresis is thus generated. The IRAP method may employ 1 or 2 primers based on different retrotransposon groups (Kalendar & Shulman, 2006). The REMAP is a method similar to IRAP; however, REMAP employs a SSR primer with a non-SSR nucleotide anchored at the 3' end of the primer (Kalendar & Shulman, 2006). IRAP and REMAP markers are actually appropriate and promising tools for phylogenetic studies, genetic mapping, genetic diversity and somaclonal variation (Kalendar et al. 1999). For instance, Bradley et al. (2011) used the IRAP marker for the detection of somaclonal variations in barley (*Hordeum vulgare*).

Current study evaluates the genetic uniformity of cassava varieties from *in vitro* propagation and estimates the possible somaclonal variation generated by *in*

vitro subcultivations. The EST-SSR, IRAP and REMAP molecular markers were used to assess the genetic variability after five and ten *in vitro* subcultures of three cassava varieties. The monitoring of subcultivations by molecular markers may be an interesting strategy in *in vitro* regeneration of plants when genetic uniformity is a commercial feature.

2. MATERIALS AND METHODS

2.1. Plant material and tissue culture

The cassava cultivars Fécula Branca, IPR-União and Olho Junto were obtained from the cassava germplasm bank maintained at the Agronomic Institute of Paraná (IAPAR, Brazil). The shoot tips (2.0 cm) were collected from the cassava plants and washed in distilled water with two drops of Tween 20, for 10 minutes. They were sterilized in a laminar airflow cabinet under aseptic conditions. The shoots from two plants of the Fécula Branca variety (FB1 and FB2), two plants of the Olho Junto variety (OJ1 and OJ2) and one plant of the IPR-União (IPRU) variety were sterilized by immersion in alcohol 50% for 1 minute, followed by 0.5% calcium hypochlorite for 5 minutes and cleaned three times in sterilized water for 2 minutes. Meristem-tips with no leaf primordia (<0.4 mm) were aseptically dissected and transferred to test tubes (15 x 2.5 cm) containing 10 ml of solidified MS medium, supplemented with 0.04 mg · L^{-1} cytokinin, 0.02 mg · L^{-1} auxin and 0.05 mg · L^{-1} gibberellin for establishment and transferred after 60 days to 0.2 mg · L^{-1} cytokinin, 0.1 mg · L^{-1} auxin and 0.05 mg · L^{-1} gibberellin for development. The FB1, FB2 and OJ1 varieties were cultivated on the culture medium supplemented with IBA auxin, whereas the IPRU and OJ2 varieties were cultivated on the culture medium with auxin NAA.

The pH of the culture media was adjusted to 5.8 before adding agar; it was autoclaved for 15 min at 121°C and 1.2 kPa. The tubes with the explants were incubated at 26±2ºC in a growth room with a 16h-photoperiod provided by white fluorescent lamps (25.3 µmol m^{-2} s^{-1}). The protocol was developed by Kuhn et al. (unpublished data). Each treatment consisted of 10 explants and data were recorded three weeks after the start of the treatments.

The meristem tips originated *in vitro* plantlets which were divided into 2 at 4 pieces (with side shoots) for fast multiplication. Therefore, one mother plant generated the cloned plants since each meristem tip was subcultured 5 (Stage 5) and 10 times (Stage 10). The plantlets were subcultured at every 40 days and 15 cloned plants from each parental plant were acclimated after five (S5) and 10 (S10) subcultures.

The plantlets generated at Stage 5 and 10 were transplanted to plastic cups with substrate (Plant Max). They were covered by cups to maintain humidity for a week; later they were taken to the green house when the cup was gradually removed for acclimatization, following Souza (2008). Leaves of the acclimated plants in the greenhouse were used for DNA extraction.

2.2. DNA extraction

Leaves from the parental plant and from 20 regenerated and acclimated plants were used for DNA extraction. Ten regenerated plants from the subculture stage 5 (S5) and 10 regenerated plants from the subculture stage 10 (S10) with a total of 21 samples from each cassava variety propagated *in vitro* were used for DNA extraction. One IPR-União clone, two Fécula Branca (FB1 and FB2) and two Olho-Junto (OJ1 and OJ2) clones, totaling 105 samples, were analyzed.

Total DNA from each plant was isolated by CTAB (cetyltrimethylammonium bromide) protocol (Knapp and Chandlee 1996), adapted by Carvalho (Cenargen-Embrapa). DNA was extracted from the young leaves of individual plants macerated in liquid nitrogen with CTAB buffer. The samples were incubated at 65ºC for two hours and centrifuged for 10 minutes. After centrifugation, the supernatant was collected and washed two times with chloroform:isoamyl alcohol (24:1). Four volumes of 100% ethanol were added to the recovered supernatant which was stored at -20ºC for one hour to precipitate the DNA. After 60 minutes of incubation the samples were centrifuged and the pellet formed was washed with cold ethanol, dried at room temperature, re-suspended in 100 µl of TE containing 4 µl of RNAse, and incubated at 4ºC overnight. After incubation, sodium acetate and ethanol were added; the samples were centrifuged and washed once more in ethanol 100%, dried at room temperature and re-suspended in 50 µl of TE for homogenization. Picodrop (Pico200 spectrophotometer) was used for the quantification of DNA.

2.3. Analysis of EST-SSR markers

Ten EST-SSR primers (Table 1) were used successfully to amplify the DNA samples of the 105 cassava plants and to estimate the somaclonal variations in the subcultures at S5 and S10.

Table 1 - Primer sequences for EST-SSR loci used in samples of cassava cultivars. EME primers designed by Kunkaew et al. (2011); MeESSR primers designed by Raji et al. (2009)

Primer	Sequence	Characteristic
EME20	F: CAGCACCAGTCAACATTCCTG	High content of dry matter
	R: CCTTCTGGCAATGAGCTCATG	
EME118	F: GCAGCATGGACATGGACC	*Xanthomonas axonopodis*
	R: GCCAAGTTAAGACCAGCAAAGC	resistance
EME212	F: GATATGGCTGCTCTTTTCATGG	High content of dry matter
	R: CCCTTCAATCTCCTCTTCAC	
EME309	F: GTAGTGATATTGGTGATCCCG	*Xanthomonas axonopodis*
	R: AACTGCACATCCGTTGACAC	resistance
EME345	F: CTGTGGCTACTCCGTTCAGTAA	High content of dry matter
	R: AGTCACCCCATTGTTCTTTGAC	
MeESSR18	F: TTAGTGGCATGGATGCAAGA	Glutathione S-Transferase
	R: GCGACTTGAACTTCCAGAAA	GST
MeESSR26	F: CGGAAATGACGAAAGAAAGG	Putative phosphatase-2c
	R: AATTCCAATTCCACCCACAC	protein
MeESSR36	F:TCGTGACGACATTGCTTCTC	UDP-D-Glucose/UDP-D-
	R: AACAACAACGGCGGAGTAAC	Galactose 4-Epimerase 2
MeESSR42	F: CTTGGGGGTCTGGGTCTAAT	Putative ATP binding
	R: TGGCATCAGAAGAACCAATG	protein
MeESSR115	F: ATTGCAAAATCCAAGCCAAC	Putative transport protein
	R: TGCCAAACAGAAACCACAAA	

2.4. DNA amplification

DNA amplification with EST-SSR primers was carried out in a volume of 20 µL: 20 ng of DNA, 1xPCR buffer (75 mM Tris-HCl; pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 2 pmol of each primer (Forward and Reverse oligonucleotides); 0.1mM of each dNTP; and 0.75 units of Platinum Taq polymerase (Invitrogen). DNA amplification was performed by Touchdown PCR program, described by Don et al. (1991). The PCR products were identified by electrophoresis in 4% agar (50% Invitrogen and 50% MetaPhor) and TBE buffer (Tris/borate 0.045 M and EDTA 0.001 M pH 8.3). The 105 cassava samples were simultaneously

analyzed by electrophoresis and the amplified segments in the gel were visualized by EtBr and UV.

2.5. EST-SSR Data Scoring and Analysis

The cassava plants with allelic variation in the *Est-SSR* loci were used to estimate the polymorphism in each cassava cultivar at subculture Stage 5 (S5) and subculture Stage 10 (S10). The 21 cassava plants of each cultivar were also scored for the presence or absence of amplified segments (1 for presence and 0 for absence of the 'homologous' segments), whilst data were entered into a binary data matrix as discrete variables. The NTSYS-pc package (Rohlf, 1989) established the relationship between the mother plant and the clones at subculture stages S5 and S10.

2.6. IRAP and REMAP amplification

The PCR reactions were performed in 25 µL volume containing 20 ng DNA, 1xPCR buffer (75 mM Tris-HCl; pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 4 pmol of each primer; 100 µL dNTP; 1 U of Platinium Taq DNA polymerase (Invitrogen). Primers were designed and tested by Kuhn et al. (unpublished data): AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5. Amplification was performed with 10 min of initial denaturation, at 95°C and 36 amplification cycles at 94°C, for 30 sec; at 55°C, for 30 sec and at 72°C, for 3 min, with a final extension of 10 min at 72°C. Whereas the primer combination AYF5xAYF9 was performed at 56°C for amplification, the combination AYF4xSSR6 (REMAP) was performed at 54°C for amplification. PCR products were identified by electrophoresis with 2% agar (agar Invitrogen:agar Metaphor; 1:3 in 1xTAE buffer followed by EtBr staining and UV visualization. Each primer was tested for yield efficiency of IRAP segments and for fingerprint quality (separate and well-definite bands in the gel).

2.7. IRAP and REMAP Data Scoring and Analysis

IRAP and REMAP bands were interpreted as 1 for presence or 0 for absence. Binary matrixes (presence/absence) were prepared from IRAP and REMAP products. Each PCR product represented a single locus. Monomorphic bands were

removed from the raw scored data sets and the weakly stained bands were not scored. The NTSYS-pc package (Rohlf, 1989) was used to establish the relationship between the mother plant and clones at subculture stages S5 and S10.

3. RESULTS AND DISCUSSION

3.1. Cassava tissue culture

The regenerated plants were successfully acclimated with the highest survival rate in subculture stage S5 (Table 2). The leaves of the acclimated plants in the greenhouse failed to show any morphological aberrations (Figure 1).

Table 2 - Acclimatization of cassava plants generated from *in vitro* culture of the clones Fécula Branca 1, Fécula Branca 2, Olho Junto 1, Olho Junto 2 and IPR-União

Clone	Acclimatized plants		Survived plants		Survival rate	
	Stage 5	Stage 10	Stage 5	Stage 10	Stage 5	Stage 10
Fécula Branca 1*	15	15	15	13	100%	86.6%
Fécula Branca 2*	15	15	14	12	93.3%	80%
Olho Junto *	15	15	13	11	86.6%	73.3%
Olho Junto **	15	15	14	11	93.3%	73.3%
IPR – União **	15	15	13	12	86.6%	80%

*Plantlets cultivated on auxin IBA.
**Plantlets cultivated on auxin ANA.

Figure 1 - Acclimated cassava plant in the greenhouse.

The acclimation was lower at development stage S10, since some plants at stage S9 developed slowly *in vitro* (Figure 2). Approximately 5% of the plantlets died at stage S9.

Figure 2 - Death of cassava plantlets at Stage 9 or have slow development.

3.2. Polymorphism in *EST-SSR* loci

Allele polymorphism was detected in only four EST-SSR (*EME20, MeESSR42, EME118, MeESSR18*) loci at stages S5 or S10 of the subcultures in the Fécula Branca (FB2 clone), Olho Junto (OJ2 clone) and IPR-União (IPRU clone) varieties (Table 3). The *EME212, EME309, EME345, MeESSR26, MeESSR36* and *MeESSR115* loci were monomorphic in the five clones of the three cassava varieties after five and ten subcultures.

Table 3 - Number of plants in the three clones (Fécula Branca 2; cultivated on IBA; Olho Junto 2 and IPR-União cultivated on NAA) with allelic variation in loci *EME20*, *MeESSR42*, *MeSSR18*, *EME118*, at the subculture stages S5 and S10

Locus	Fécula Branca 2		Olho Junto 2		IPR União		
	S5	S10	S5	S10	S5	S10	Overall
EME20	-	-	2	3	-	-	5
MeESSR42	-	-	-	-	-	2	2
EME118	-	1	-	2	1	-	4
MeESSR18	-	1	-	-	-	3	4
Total	0	2	2	5	1	5	15

Reduced polymorphism in SSR loci of micropropagated plantlets derived from vegetative buds of *Populus tremuloides* was also reported in the investigations by Rahman and Rajora (2001). Only one SSR primer revealed somaclonal variation in 1 out of the 13 plantlets obtained from one genotype, while other primers revealed somaclonal variations in one out of four plantlets from another genotype. The SSR

markers have revealed low genetic diversity and reduced somaclonal variation in plants generate from meristem cultures (Agrawal et al., 2014). Gao et al. (2009) also reported that some SSR markers in the genome showed a polymorphism which was higher than other SSRs in the somaclonal variation analysis in rice.

A lower number of survival plants with higher allelic variations was observed at S10 rather than at S5 of subcultures in micropropagated plants of FB2, OJ2, and IPRU clones. The most polymorphic locus was *EME18* since it showed allelic variation in the three cassava varieties whilst the highest number of plants with somaclonal variation (5 plants) was detected in *EME20* locus. The *EME20* locus was polymorphic only in the micropropagated plants from OJ2 clone. The OJ2 clone showed the highest number of plants with allelic variations (7 plants) at S5 and S10 of subcultures, while the lowest polymorphism in the *EST-SSR* loci was reported in the FB2 clone. FB2 clone plants were genetically uniform at S5 of the subculture and only two plants showed allelic variation at S10 of subculture. Since FB1 and OJ1 clones showed no allelic variation in 10 *EST-SSR* loci analyzed in current study, the Fécula Branca variety may be characterized as the most genetically stable variety at S5 and S10 of subcultures. On the other hand, the Olho-Junto (OJ1 and OJ2 micropropagated plants) and IPR-União varieties may be characterized as both genetically unstable at S5 of the subculture, whilst the IPR-União variety showed the highest genetic instability at S10 of subculture.

The relative genetic instability observed in clones from the Olho Junto and IPR-União varieties contrasts with the genetic stability reported to others cassava cultivars micropropagated subjected to slow growth for 10 years under similar conditions in culture medium containing the same growth regulators (Roca et al., 1994). However, the genetic stability reported by Roca et al. (1994) in their study with seven cassava varieties using molecular techniques may be due to reduced number of samples (only three plants) used to available the somaclonal variation induced in vitro or due to the genotype specificity. Genotype specificity in in vitro propagation was evident in sugarcane varieties maintained by conventional bud propagation (via rhizomes) and by in vitro shoot tip cultures (Silva et al., 2008). In vitro multiplication of four varieties increases genetic variability, while in vitro multiplication of other four leads to genetic similarity.

In our investigation, the Fécula Branca variety was characterized as the most genetically stable variety in the S5 and S10 stages of subcultures. The individual

analysis of FB2 micropropagated plants by the UPGMA method in the NTSYS 2.02 program (Rohlf, 1989) showed that 95% of the plants formed a group with full genetic identity with the parental plant; only one plant belonged to an isolated group in the dendrogram based on the arithmetic complement of the Jaccard index (Figure 3)

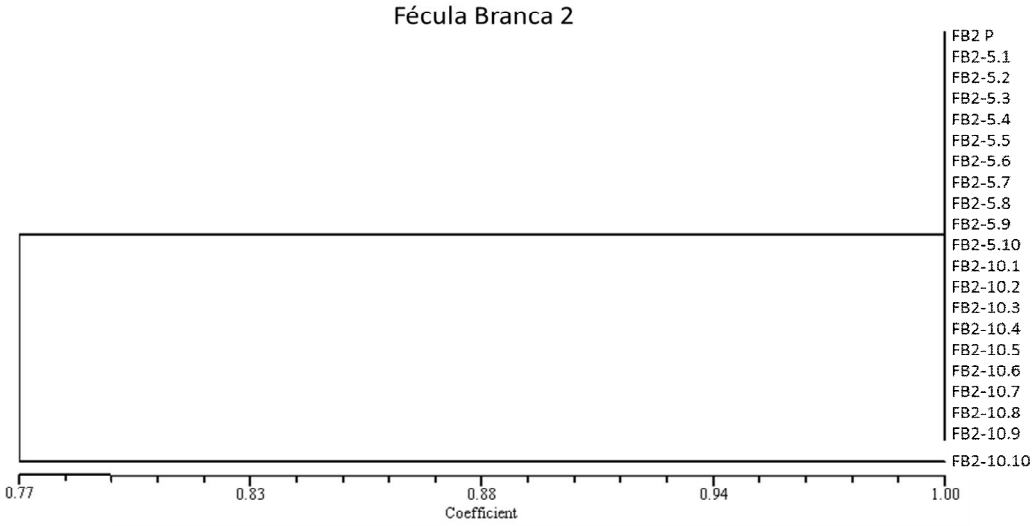


Figure 3 - Dendrogram of the variety Fécula Branca 2, based on UPGMA coefficient for EST-SSR data.

Further, 75% of the OJ2 micropropagated plants formed a group with full genetic identity with the parental plant; the second group was formed by two plants at S5 of subculture and one plant at S10 of subculture; the third group was constituted by two plants at S10 of subculture (Figure 4)

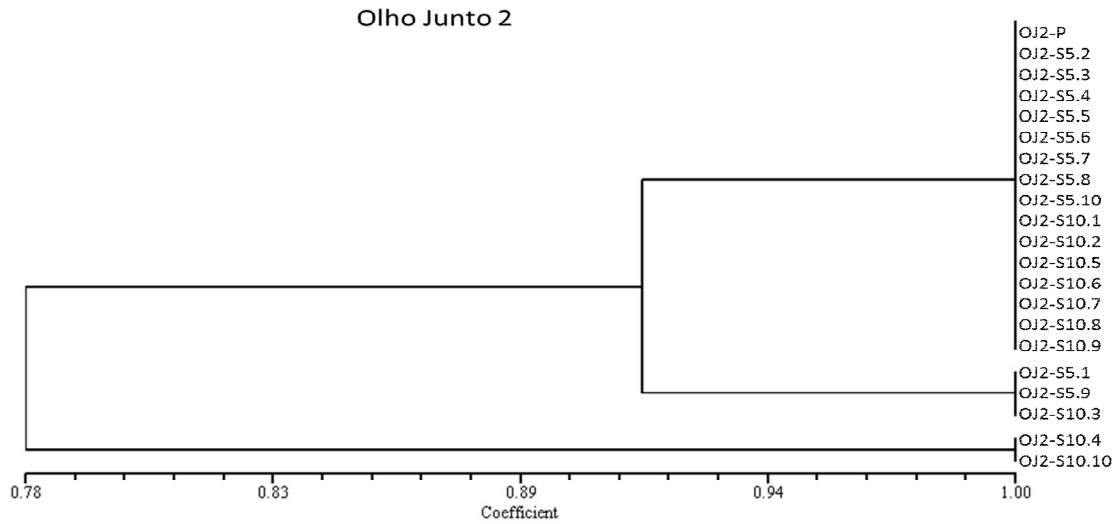


Figure 4 - Dendrogram of variety Olho-Junto 2, based on UPGMA coefficient for EST-SSR data.

Only 70% of the plants in the IPR-União variety showed genetic identity with the parental plant. The dendrogram revealed that the micropropagated plants from IPR-União were divided into four groups (Figure 5). The first group with 70% of identical plants; the second group formed by three plants at S10 of subculture with less than 10% of genetic divergence of the plants of the first group; the third group was also formed by two plants at S10 of subculture; the fourth group was formed by only one plant at S5 of subculture.

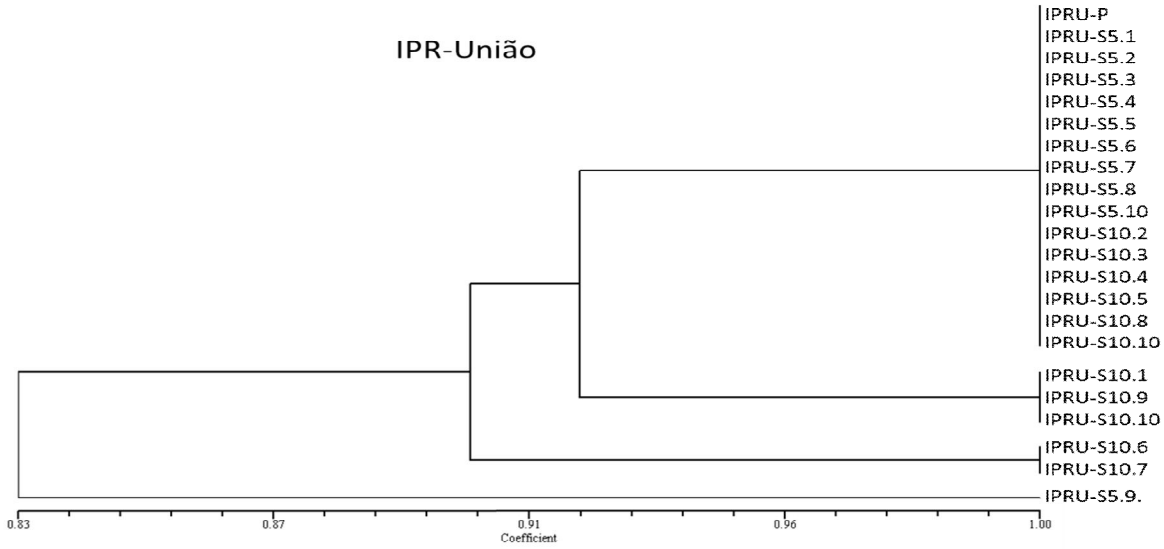


Figure 5 - Dendrogram of the variety IPR-União, based on UPGMA coefficient for EST-SSR data.

3.3. IRAP and REMAP polymorphism

The six primer combination IRAP and REMAP (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, and AYF4 x SSR5) yielded 72 bands in the five micropropagated plant clones (FB1, FB2, OJ1, OJ2, and IPRU) from three cassava varieties (Fécula Branca, Ollho-Junto and IPR-União). The primers AYF2xAYF3 and AYF4 x SSR6 amplified the largest number (16) and the smallest number (9) of alleles respectively. Figure 6 illustrates the polymorphism generated by the AYF3 x AYF8 primer in the FB1 micropropagated plants, indicating occurrence of somaclonal variation during the subculture stages (S5 and S10).

The activation of transposable elements is a source of chromosome-based somaclonal variation. The tissue culture provides a favorable environment for DNA sequence transposition; the transposons activation may activate genes or silence

putative genes which affect the protein synthesis in the plant and its development (Bairu et al. 2011).

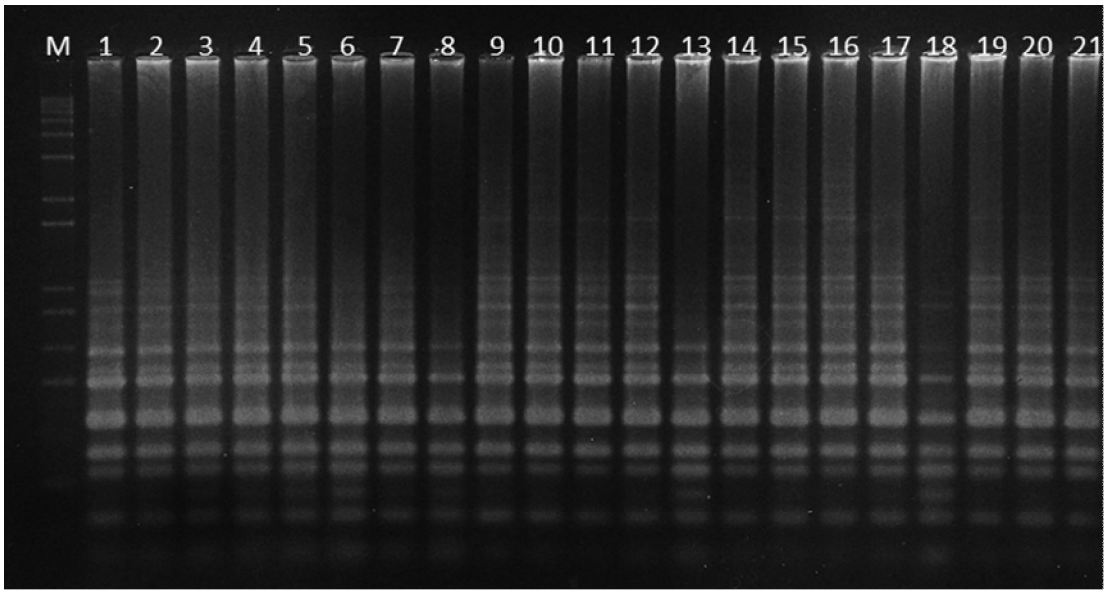


Figure 6 – IRAP gels from the Fécula Branca 2 variety, showing polymorphism with the AYF3 x AYF8 primer. M: 1kb DNA Marker. Sample 1: parental plant. Samples 2-11 plants at Stage 5. Samples 12-21 plants at Stage 10.

The polymorphism of IRAP and REMAP markers (AYF8, AYF9, AYF2 x AYF3, AYF3 x AYF8, AYF5 x AYF9, AYF4 x SSR5) in the cassava varieties (Fécula Branca, Olho Junto and IPR-União) was higher at S10 of the subcultures than at S5 (Tables 4 and 5).

Table 4- Number of polymorphic segments of DNA and polymorphism (P) of markers AYF8, AYF2 x AYF3, AYF5 x AYF6, AYF3 x AYF8, AYF4 x SSR6 and AYF9 in the cassava varieties Fécula Branca (FB1 and FB2), IPR-União (IPRU) and Olho Junto (OJ1 and OJ2), at S5 of the subculture

Primer	TNS	FB1	FB2	IPRU	OJ1	OJ2
AYF8	11	0	1	5	3	3
AYF2 x AYF3	16	6	4	3	3	3
AYF5 x AYF6	11	4	1	1	2	2
AYF3 x AYF8	12	1	5	0	0	1
AYF4 x SSR6	9	0	1	0	0	0
AYF9	13	0	3	1	2	0
Total	**72**	**11**	**15**	**10**	**10**	**9**
%P		**15.27**	**20.83**	**13.88**	**13.88**	**12.5**

TNS: Total number of amplified segments.

In the Fécula Branca variety (FB1 and FB2 clones) the highest polymorphism was observed at S5 (36.11%) and S10 (80.5%) of the subculture.The lowest polymorphism of IRAP and REMAP markers was observed at S5 (13.8%) and S10 (40.27%) of the subcultures of the IPRU clone.

The lowest polymorphism of IRAP and REMAP markers in the IPRU clone indicates the occurrence of less somaclonal variation in the IPRU clone than in FB1, FB2, OJ1 and OJ2 micropropagated clones. In contrast, the occurrences of somaclonal variation seem higher in FB1 and FB2 clones.

Table 5 - Number of polymorphic segments of DNA and polymorphism (P) of markers AYF8, AYF2 x AYF3, AYF5 x AYF6, AYF3 x AYF8, AYF4 x SSR6 and AYF9 in the cassava varieties Fécula Branca (FB1 and FB2 clones), IPR-União (IPRU) and Olho Junto (OJ1 and OJ2 clones) at S10 of the subculture

Primer	TNS	FB1	FB2	IPRU	OJ1	OJ2
AYF8	11	4	4	6	4	7
AYF2 x AYF3	16	9	7	8	4	5
AYF5 x AYF6	11	6	3	2	2	4
AYF3 x AYF8	12	3	5	1	0	5
AYF4 x SSR6	9	6	4	2	0	5
AYF9	13	4	3	10	3	3
Total	**72**	**32**	**26**	**29**	**13**	**29**
%P		**44.44**	**36.11**	**40.27**	**18.05**	**40.27**

TNS: Total number of amplified segments.

The highest polymorphism at S10 of the subculture seems to be a non-cumulative event since FB2 clones showed the highest polymorphism at S5 of the subculture whilst the polymorphism was higher in FB1 clone at S10. Likewise, the OJ1 clone showed the highest polymorphism at S5 of the subculture, while polymorphism was higher in OJ2 clone at S10. The differential polymorphism evident in the clones from the Fécula Branca (FB1 and FB2) and Olho Junto (OJ1 and OJ2) varieties may be a device to genetic variability within each variety. Thus, the genetic variability within each variety may determine the global level of somaclonal variation generated in the variety and make difficult the predictions for the somaclonal variations for the different subculture stages. In our experiments, the OJ2 clone is considered to be the most stable (less polymorphism in the IRAP and REMAP

markers) at S5 of subculture, while the OJ1 clone seems to be more stable at S10 of the subculture. Differential response genotypes dependent on meristem micropropagation have been reported in different varieties of sugarcane (Zucchi et al., 2002), although the different rates of polymorphism had no direct association with the subculture stage.

The dendrograms based on the arithmetic complement of the Jaccard index (Rohlf, 1989) revealed the formation of two well-defined groups which comprised clones generated at S5 and S10 of the subculture as well as the highest genetic diversity in clones generated at S10 (Figure 7). The greatest genetic stability in the OJ2 clone at S5 of the subculture is evident in Figure 7D which shows the genetic identity (I = 1.00) in seven regenerated plants, while the greatest genetic stability in the OJ1 clone (0.87 ≥ I ≤ 1.00) is evident at S10 of the subculture (Figure 7C). Plants with genetic identity at S10 of the subculture lower than the genetic identity detected in OJ1 clone may be observed in FB1 (0.64), FB2 (0.75), OJ2 (0.63) and IPRU (0.71) clones (Figures 7A, 7B, 7D and 7E).

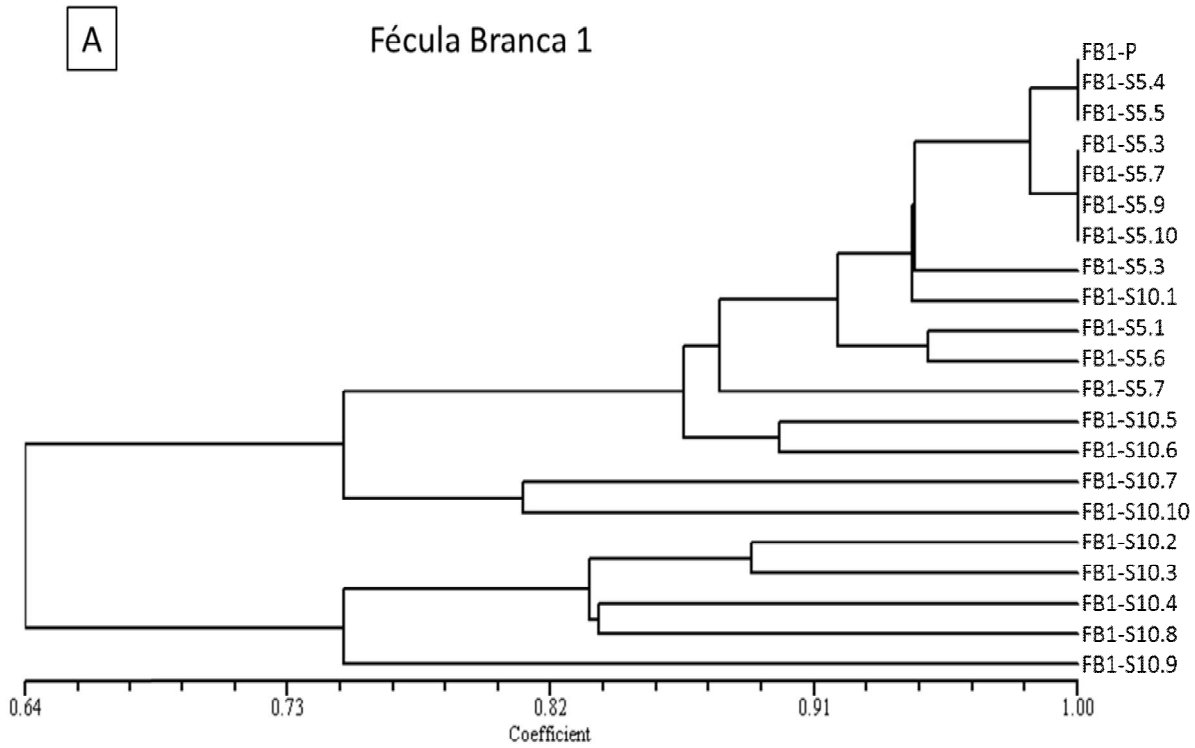
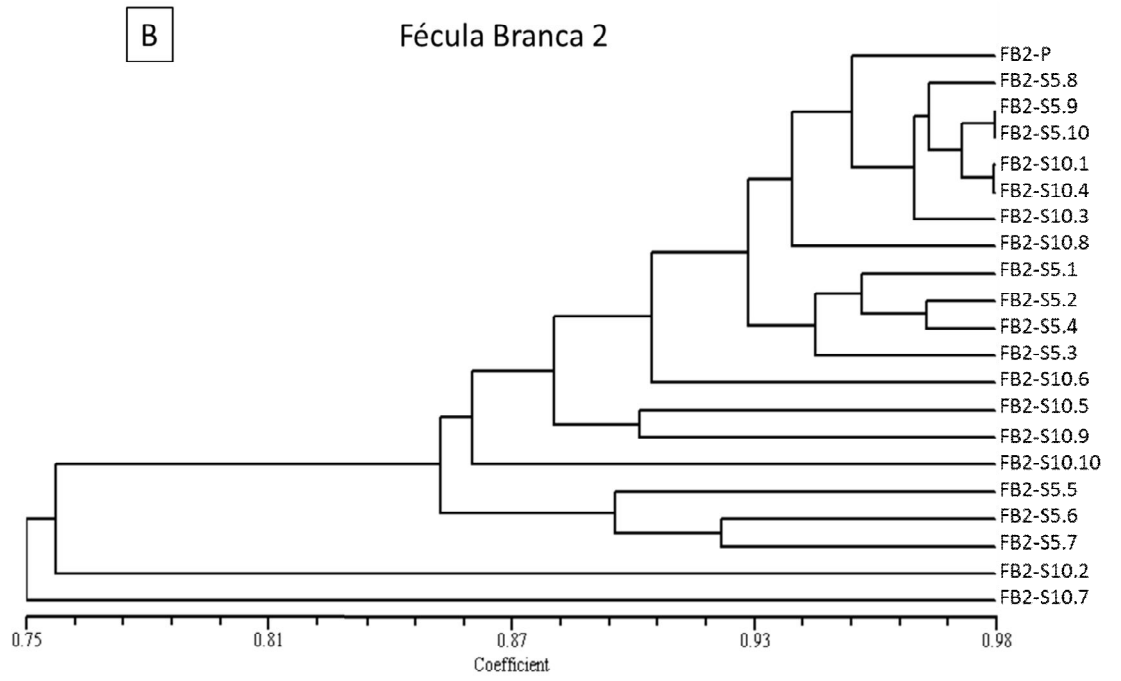


Figura 7, cont.



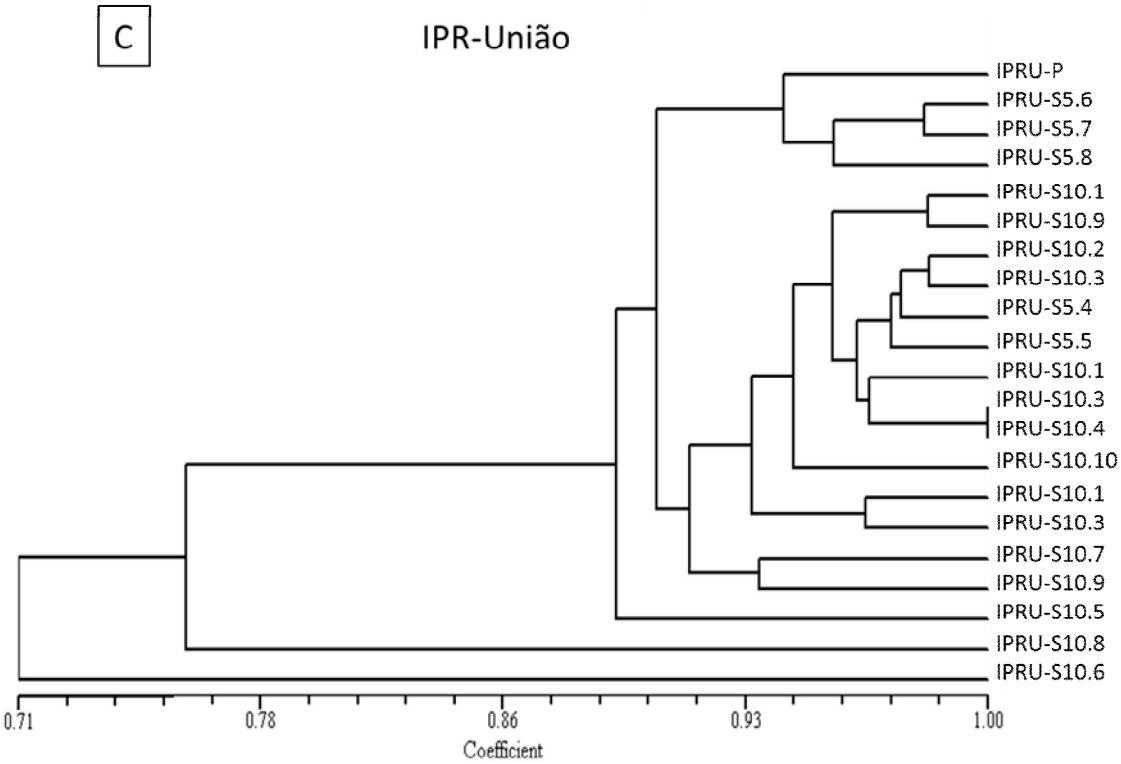


Figura 7, cont.

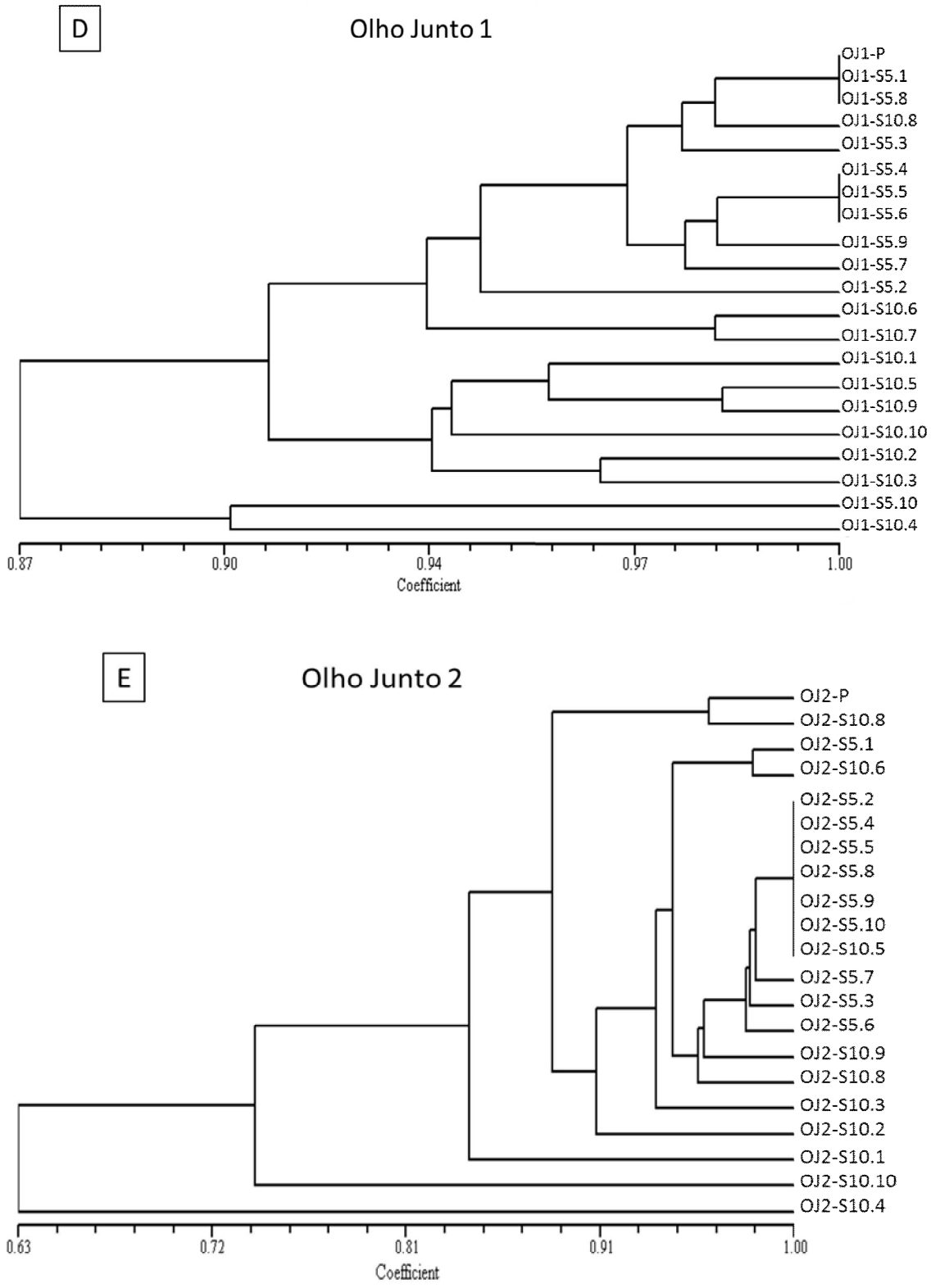


Figure 7 - 0 Dendrograms of clones: A: Fécula Branca 1; B: Fécula Branca 2; C: IPR-União; D: Olho Junto 1 and E: Olho Junto 2, generated at S5 and S10 of subculture, based on coefficient UPGMA for IRAP and REMAP data.

3.4. Polymorphism of EST-SSR and IRAP/REMAP markers in cassava clones

Polymorphisms detected by IRAP/REMAP markers in clones of Fécula Branca, Olho Junto and IPR-União cassava varieties were higher than the polymorphism revealed by EST-SSR markers at S5 and S10 of the subcultures (Table 6). The IRAP/REMAP markers revealed a global genetic diversity more than three times higher than the EST-SSR markers in the three cassava cultivars. The absence of genetic diversity at *EST-SSR* loci in the FB1 and OJ1 clones indicating genetic stability contrasts with the high genetic diversity revealed by IRAP/REMAP primers.

Table 6 - Polymorphisms detected by EST-SSR and IRAP/REMAP markers in clones of the cassava varieties Fécula Branca, IPR-União, and Olho Junto available at S5 and S10 of the subcultures

Clone	Level of polymorphism at S5	Level of polymorphism at S10
Fécula Branca 1		
IRAP/REMAP	15.3%	47.2%
EST-SSR	0	0
Fécula Branca 2		
IRAP/REMAP	17.8%	43%
EST-SSR	0	10%
IPR-União		
IRAP/REMAP	13.9%	41.7%
EST-SSR	10%	20%
OLHO JUNTO 1		
IRAP/REMAP	12.5%	18%
EST-SSR	0	0
Olho Junto 2		
IRAP/REMAP	11.1%	40.3%
EST-SSR	10%	20%
Mean:		
IRAP/REMAP	14.12%	38%
EST-SSR	4%	10%

Due to inherent mobility of retrotransposons and their possible induction for changes in the genome under stress conditions caused by *in vitro* culture (Hirochika, 1993; Snustad and Simmons, 2011; Bradley, 2011), a higher polymorphism by IRAP and REMAP markers in *in vitro* propagated cassava plants was expected. Contrastingly, in EST-SSR loci containing expressed sequences tags, the expected genetic diversity is lower since variations in the EST-SSR loci may result in the metabolic changes of the genomes.

The locus *EME18* associated with expressed sequences to *Xanthomonas axonopodis* resistance protein (Table 2) was the most polymorphic with allelic variations in the three cassava varieties (Table 4). The allele variation in *EME18* locus points out a possible divergence between the products of these loci in the three FB2, OJ2, and IPRU cassava clones during the stages of subcultures. The highest number of plants with allelic variation at the *EME20* locus (Table 4) draws attention to a possible variation in proteins related to the high content of dry matter in OJ2 clone (Table 4). Likewise, the allelic variation in the *MeESSR42* locus only at S10 of the subculture of the IPR-União clone may indicate a variation in a putative ATP binding protein. The ATP binding proteins have a binding site that allows ATP molecule to interact. In fact, it has an important role in the release of energy and in the control of metabolic pathways (Bustamante et al., 2004).

The locus *MeESSR18* associated with expressed sequences to Glutathione S-Transferase isozymes (GST; EC 2.5.1.18) was polymorphic in the FB2 and IPR-União clones only at S10 of the subculture. The GST isozymes are enzymes that catalyze the conjugation of glutathione (GSH) to a variety of cytotoxic substrates showing their ability in detoxifying herbicides and conferring herbicide tolerance (review in Marrs, 1996). GST has also been related to stress responses such as pathogen attack, oxidative stress and heavy-metal toxicity, and in addition, as carriers of natural auxin (Bilang and Sturm, 1995). The important function of these proteins and enzymes in cell metabolism may explain the highest genetic variability of the loci responsible for their synthesis.

DNA polymorphism in SSR loci lower than the polymorphism revealed by transposons markers has been related in other micropropagated species. Absence of somaclonal variation was detected for polyploidy plants of *Asparagus officinalis* L. obtained by the culture of rhizome bud explants with the EST-SSR marker (Regalado et al. 2015). Higher polymorphism was also revealed by the marker TD based on

transposon (Transposon Display) than with SSR markers in rice (Gao et al., 2009). Since all somaclones mutants of rice showed polymorphism when analyzed by Transposon Display, the insertions of transposons during tissue culture may play important roles in the induction of somaclonal variation.

The IRAP/REMAP markers used in current study to evaluate the induced variation in DNA level of cassava varieties also revealed polymorphism in all cassava clones. EST-SSR markers indicated genetic stability in one clone of the Fécula Branca and Olho Junto varieties. The use of molecular markers to monitor micropropagation from apical meristems in cassava varieties indicated the occurrence of somaclonal variation and indicated that IRAP and REMAP markers were more effective than EST-SSR markers to evidence the *in vitro*-induced genetic variability. Although a higher genetic stability has been expected in micropropagated plants using apical or nodal meristems as explants (Bairu et al., 2011), and reported (Zucchi et al., 2002; Ray et al., 2006; Borse et al., 2011; Rambaud et al. 2013; Punyarani et al., 2013; Prasad et al., 2015; Agarwal et al., 2015) in micropropagated using apical or nodal meristem explants, however the use of IRAP and REMAP markers showed polymorphisms higher than 40% in four cassava clones of Fécula Branca, IRP-União and Olho Junto varieties. Moreover, the IRAP and REMAP markers also demonstrated that the highest or lowest somaclonal variations induced in the cassava clones depended on the genetic variability within each cassava variety. Cassava varieties with low genetic diversity may produce more genetically uniform clones using apical meristems as explants from the different plants.

4. CONCLUSIONS

a) The use of molecular markers to monitor micropropagation from apical meristems in cassava varieties indicated the occurrence of somaclonal variation and appointed IRAP and REMAP markers as more effective than EST-SSR markers to evidence the genetic variability induced *in vitro*;

b) The highest or lowest somaclonal variations induced in the cassava clones depended on the genetic variability within each cassava variety.

c) IRAP and REMAP are more efficient than EST-SSR, however the molecular marker based on EST is more is more specific and shows variation in a expressed sequence.

5. CONCLUSÕES GERAIS

a) A propagação das plantas de mandioca *in vitro* a partir de meristemas apicais é um procedimento relativamente simples. Entretanto, apenas três variedades foram cultivadas com sucesso: as variedades Fécula Branca, IPR-União e Olho Junto, indicando que adaptações devem ser feitas às concentrações dos reguladores de crescimento para proporcionar o desenvolvimento das variedades Baianinha, Icaraíma, Pioneira e Tamboara.

b) Os meristemas apicais da variedade Fécula Branca apresentaram melhor desenvolvimento em meio de cultura suplementado com o regulador de crescimento IBA; os meristemas apicais das variedades Olho Junto se desenvolveram igualmente em meio de cultura suplementado com o regulador IBA e com o regulador NAA; e a variedade IPR-União se desenvolveu de maneira satisfatória em meio de cultura suplementado com o regulador de crescimento NAA. Independente das diferentes auxinas utilizadas para o cultivo dos explantes, a citocinina 6BA e a giberelina GA_3 foram indispensáveis em todas as fases do cultivo e em todas as variedades de mandioca.

c) A propagação *in vitro* da mandioca (*Manihot esculenta*) é dependente do genótipo e também da diversidade genética de cada variedade.

d) Foram desenhados 12 *primers* baseados nas sequencias LTR dos retrotransposons. Estes *primers* formaram 42 combinações (IRAP) e amplificaram 431 segmentos de DNA. Os *primers* LTR foram combinados também com sequências SSR (REMAP).

e) Os *primers* IRAP e REMAP desenhados para este estudo foram eficientes para a análise da diversidade genética, diferenciando quatro variedades de mandioca. Este marcador é uma ferramenta importante para a análise da estrutura e da diversidade da cultura da mandioca, devido ao fato de que os retrotransposons são abundantes no seu genoma.

f) As três variedades analisadas no capítulo I usando os marcadores IRAP e REMAP foram divididas em dois grupos: a variedade Olho Junto apareceu em um grupo isolado, enquanto as variedades Fécula Branca e IPR-União foram agrupadas no mesmo grupo. No capitulo II, diferentes amostras das mesmas variedades foram coletadas para a análise molecular e estas foram divididas de maneira similar: as

variedades IPR-União, Fécula Branca e Tamboara formaram um grupo, enquanto a variedade Olho Junto continuou isolada, demonstrando a reprodutibilidade e confiabilidade dos marcadores baseados em retrotransposons IRAP e REMAP.

g) Os marcadores baseados em retrotransposons IRAP e REMAP identificaram polimorfismo em clones de todas as variedades de mandioca cultivadas *in vitro*, enquanto o marcador EST-SSR identificou polimorfismo em clones de apenas 3 variedades, indicando que o uso dos marcadores IRAP e REMAP foi mais eficiente para a análise da variação somaclonal do que os marcadores EST-SSR.

h) As plântulas cultivadas *in vitro* até o estágio 5 de subcultivo (S5) apresentaram um índice de polimorfismo inferior às plantas cultivadas até o estágio 10 (S10), demonstrando que a taxa de variação somaclonal aumenta conforme as plântulas são subcultivadas.

i) O índice de variação somaclonal é dependente da variabilidade genética dentro de cada variedade de mandioca.

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