

ALTERNATIVAS PARA LA PRODUCCIÓN DE CARDENÓLIDOS MEDIANTE TÉCNICAS BIOTECNOLÓGICAS EN DIGITALIS SPP

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- ✓ **Elio Jiménez González (20%)**: Participó en el diseño de experimentos, la interpretación de los resultados y en la revisión de las publicaciones.
- ✓ **Borys Chong Pérez (19%)**: Participó en el diseño y montaje de los experimentos relacionados con la transformación genética e interpretación de los resultados obtenidos. Participó en la escritura de las publicaciones y del documento.
- ✓ **Alina Capote Pérez (18%)**: Participó en el diseño y montaje de los experimentos además de la interpretación de los resultados. Mantuvo el banco de plantas de *Digitalis*.

- ✓ **Anabel Pérez Pérez** (15%): Participó en el montaje de los experimentos y mantuvo el banco de plantas de *Digitalis*.
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Resumen

Las plantas de *Digitalis lanata* Ehrh. y *Digitalis purpurea* L. son de interés económico debido a que constituyen las únicas fuentes de cardenólidos, fármacos irremplazables en tratamientos de insuficiencia cardíaca. Las enfermedades cardiovasculares afectan la calidad de vida de un sinnúmero de cubanos y constituyen la segunda causa de muerte en el país. La síntesis por vía química de estos compuestos es inviable, de manera que las estrategias se han visto restringidas al aumento de la productividad de las plantas y pudiera tenerse en cuenta para la sustitución de importaciones. El presente trabajo aborda un grupo de resultados científicos novedosos y de interés práctico sobre la producción de cardenólidos mediante técnicas biotecnológicas. Se desarrolló el escalado para producir biomasa en sistemas de inmersión temporal (SIT) los cuales constituyen un método efectivo para producir biomasa de *D. purpurea* y *D. lanata* a gran escala, resultado que permitió diseñar un esquema de producción como estrategia para obtener cardenólidos in vitro. Se demostró que la elicitación incrementó el contenido de cardenólidos en brotes de *D. lanata* en SIT. Los mejores resultados en cuanto a contenido de cardenólidos se alcanzaron con 0,1 g.L⁻¹ de ChitoPlant®, donde se incrementó 3,22 veces el lanatósido C y 1,57 la digoxina respecto al control sin elicitar. Por último, se describe un protocolo para la transformación genética mediado por *Agrobacterium tumefaciens* a partir de explantes foliares, no referido hasta el momento en la literatura internacional. Se detectó la presencia de los transgenes mediante PCR y la hibridación por Southern. La integración de los resultados científicos obtenidos permitirá disponer de la base metodológica para el desarrollo de tecnologías para producir cardenólidos in vitro.

Comunicación Corta

El género *Digitalis* agrupa alrededor de 20 especies de interés ornamental y medicinal entre las que se destacan *Digitalis purpurea* L. y *Digitalis lanata* Ehrh que contienen cardenólidos. Estos compuestos son usados para el tratamiento de la insuficiencia cardíaca (Hornberger *et al.*, 2000). Además, se han descrito sus efectos en el control del cáncer de riñón, de próstata y leucemia (Lewis, 2009). En Cuba, la planta no se cultiva comercialmente y la síntesis química del compuesto no es económicamente viable por lo que se importa la materia prima proveniente de plantas de *Digitalis* de la India. Es por esto que existe una demanda creciente de tecnologías de producción alternativas al cultivo en condiciones naturales. Con anterioridad, en el 2008, nuestro grupo de trabajo presentó resultados relacionados con la producción de metabolitos secundarios en especies medicinales tales como *Digitalis purpurea*. Dichos resultados no se presentan en esta propuesta de premio y sirvieron de base para los que aquí presentamos a continuación.

Producción de biomasa a gran escala de las especies *D. purpurea* y *D. lanata* en sistemas de inmersión temporal

Se describe un método eficiente para la producción de biomasa de *D. purpurea* y *D. lanata* a gran escala en sistemas de inmersión temporal. La mayor producción de biomasa a menor escala por SIT de 1,0L de capacidad 104,03 gMF (gramos de masa fresca) y 5,74 gMS (gramos de masa seca) se obtuvo cuando se inocularon 12 explantes y con una inmersión de dos minutos cada 4 h. Se obtuvo una producción neta de cardenólidos (digitoxina 167,6 µg y digoxina 119,9 µg) por SIT. Sobre la base de estos resultados se diseñó un esquema de producción de biomasa en los SIT de 5 L para evaluar sus potencialidades en las especies *D. lanata* y *D. purpurea* como una etapa fundamental en la obtención de cardenólidos mediante el cultivo *in vitro*. A modo de comparación es posible señalar que, durante el escalado, para la densidad de inóculo, medio de cultivo así como capacidad del frasco de cultivo se mantuvo una relación proporcional de 1:5 con relación a los frascos de 1,0 L que se utilizaron para los experimentos previos. Esta relación nos indica que el escalado resultó significativo en cuanto a la producción de biomasa aún cuando la producción de cardenólidos pudiera incrementarse para hacer esta estrategia económicamente atractiva. No obstante, existen otras potencialidades de los SIT que justifican su aplicación para la producción de biomasa con fines farmacológicos. Las mismas, están dadas por la posibilidad de producir grandes cantidades de biomasa en condiciones ambientales controladas en cualquier época del año; mientras que, la cosecha en condiciones de campo se realiza en el segundo año del ciclo de vida de la planta antes de la floración. Además la biomasa está libre de contaminantes y se obtiene una producción uniforme que garantiza la calidad del compuesto. El proceso de extracción puede ser más simple, rápido y eficiente comparado con la extracción a partir de plantas cultivadas en condiciones de campo. Hasta el momento, no se describe en la literatura el empleo de los SIT en el género *Digitalis* y este constituye el primer informe para la producción de biomasa y cardenólidos (Plant Cell Tissue and Organ Cult 2009 99:151-156 FI 1,271).

Incremento de la producción de cardenólidos mediante la adición de elicitores en brotes de *Digitalis lanata* en sistemas de inmersión temporal

En *Digitalis* se conoce que la síntesis de cardenólidos ocurre como un mecanismo de defensa de la planta ante el ataque de insectos (Wink, 2010). Esto hace que sea posible incrementar la biosíntesis de cardenólidos mediante ciertos factores que causen estrés e induzcan dichos mecanismos. Se estudió el efecto del Chitoplant®, Silioplant® y jasmonato de metilo en la producción de biomasa y cardenólidos en brotes de *D. lanata* en sistemas de inmersión temporal. Se demostró que fue posible incrementar el contenido de cardenólidos en la biomasa producida. La mayor acumulación de lanatósido C se obtuvo con Chitoplant® (0,1 g.L⁻¹), obteniéndose 316 µg g-MS⁻¹ y con Silioplant® (0,01 g.L⁻¹) (310 µg g-MS⁻¹), para un incremento de 2,2 veces comparado con el cultivo sin elicitar. La elicitación de brotes en SIT resultó en un estrés oxidativo caracterizado por un incremento en el contenido de peróxido de hidrógeno y malondialdehído. Esto apunta a una relación entre la peroxidación lipídica, contenido de peróxido de hidrógeno y acumulación de lanatósidos C. Como estrategia para estimular la síntesis de metabolitos secundarios, la elicitación ha tenido una marcada aplicación comercial en los últimos años. A esto se le adiciona, las ventajas de los SIT que son obvias para este propósito además de permitir la variación de las

condiciones de cultivo y que los elicitores sean añadidos fácilmente al medio de cultivo. Además permitirá el estudio de genes relacionados con la biosíntesis de cardenólidos mediante la evaluación de la expresión de los mismos ante el efecto del elicitor. El empleo simultáneo de estas dos estrategias, es una variante muy atractiva para la biosíntesis y acumulación de cardenólidos en el género *Digitalis*. (Plant Cell Tissue and Organ Cult 2012 110:153-162 FI 3,633).

Transformación genética de *D. purpurea* mediada por *Agrobacterium tumefaciens*

El presente estudio describe un protocolo eficiente de regeneración vía organogénesis indirecta a partir de explantes foliares y transformación genética mediada por *Agrobacterium tumefaciens* en *Digitalis purpurea* L. Se determinaron las concentraciones mínimas inhibitorias de geneticina (70 mg.L⁻¹) y de higromicina B durante la formación de callos (12 mg.L⁻¹) y esta última en la multiplicación de brotes (75 mg.L⁻¹). Se transformaron segmentos de hojas con las cepas EHA105 y C58C1-pMP90, con el plasmidio pTJK136 que contenía los genes *nptII* y *uidA*. Con ambas cepas se obtuvieron altos niveles de expresión transitoria del gen *uidA*, aunque sin diferencias significativas entre ellas. Sin embargo, a partir de la transformación con la cepa C58C1-pMP90 la formación de callos y la regeneración de plantas fueron significativamente mayores respecto a la EHA105. La presencia de los transgenes fue detectada en las líneas de plantas regeneradas mediante PCR y se confirmó la integración estable del gen *nptII* mediante la hibridación de *Southern*. Se obtuvo un promedio de 6,91 líneas transgénicas por explante inicial inoculado con la C58C1Rif^R (pMP90). Hasta la fecha, se han publicado muy pocos trabajos acerca de la transformación genética en el género *Digitalis* y este es el primer protocolo descrito en *D. purpurea*. El protocolo para la transformación genética descrito podrá contribuir a estudios funcionales para un mejor entendimiento de las rutas biosintéticas y la ingeniería metabólica de los cardenólidos para el desarrollo de genotipos de alta productividad. En el futuro, se requerirá de estudios relacionados con los mecanismos de síntesis de cardenólidos e identificación de enzimas específicas que permitan un incremento en la eficiencia de las alternativas estudiadas. (Biotecnología vegetal 2008, 8: 115–118; Biotecnología Vegetal 2013, 13:23-31; Journal Biotechnology 2010 Supplement 1. DOI: 10.1016/j.jbiotec.2010.09.722 (FI 3,05); Plant Biotechnology Reports DOI 10.1007/s11816-014-0329-0 (FI 1,59).

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La novedad e impacto científico de los resultados

Novedad Científica:

Se describe el empleo de los sistemas de inmersión temporal para la producción de biomasa y de cardenólidos en *Digitalis purpurea* L. y *D. lanata*, el cual constituye el primer informe hasta el momento a nivel internacional para el género. Además, se demuestran las potencialidades de los mismos como alternativa al cultivo en condiciones naturales. También se informa por primera vez resultados acerca del empleo de elicitores para estimular la síntesis de cardenólidos en brotes cultivados *in vitro* de *D. lanata* en sistemas de inmersión temporal. Se describe el primer protocolo para la transformación genética mediada por *Agrobacterium tumefaciens* en *Digitalis purpurea*, resultados sin precedentes en la literatura científica que pudieran emplearse en programas de mejoramiento genético de esta especie para aumentar la producción de cardenólidos.

Beneficios alcanzados: Se estableció un método para la producción de biomasa a partir de brotes de *Digitalis* basado en sistemas de inmersión temporal y el uso de la elicitación que servirán de base para los procesos de síntesis y acumulación de metabolitos de interés farmacéutico. El protocolo para la transformación genética mediado por *Agrobacterium tumefaciens* descrito puede emplearse en estudios funcionales de genes específicos de la ruta. También constituyen una herramienta útil como modelo experimental para el estudio de la biosíntesis de cardenólidos. Estos estudios contribuirán a un mayor conocimiento de las rutas metabólicas e identificación

de nuevos genes y enzimas que la regulan y por consiguiente lograr un mayor incremento de la producción *in vitro* de cardenólidos. A su vez, las estrategias desarrolladas pudieran combinarse para lograr un incremento en la producción de cardenólidos en el género *Digitalis* y podrán ser empleadas para el desarrollo de tecnologías para la producción *in vitro* de cardenólidos a escala comercial. En Cuba, las enfermedades cardiovasculares afectan la calidad de vida de un sinnúmero de cubanos y constituyen la segunda causa de muerte en el país.

Actualmente se consideran los digitálicos la tercera línea de tratamiento de la Insuficiencia Cardíaca Sistólica. Si tomamos en cuenta que en el país el 13,1% de la población está constituida por gerontes y a ellos agregamos el aumento de la esperanza de vida, es posible plantear que estos medicamentos seguirán teniendo un uso frecuente en la población. Su síntesis por vía química es inviable, de manera que las estrategias se han visto restringidas al aumento de la productividad de las plantas y pudiera tenerse en cuenta para la sustitución de importaciones. La obtención de genotipos de *Digitalis* con elevada producción de glucósidos cardiotónicos mediante el cultivo *in vitro*, facilitaría la producción de estos compuestos en condiciones controladas en un tiempo relativamente corto y a través de una tecnología limpia. De esta manera se evitan los riesgos de la producción en campo y se incrementa la calidad, uniformidad y rendimiento del producto. Permitiría además establecer sistemas de producción definidos con estricto control de la calidad, así como disponer del producto independientemente de los cambios ambientales.

Publicaciones realizadas que guardan relación con el presente trabajo:

- ✓ Pérez-Alonso N, Chong-Pérez B, Capote A, Pérez A, Izquierdo Y, Angenon G, Jiménez E (2014) *Agrobacterium tumefaciens*-mediated genetic transformation of *Digitalis purpurea* L. Plant Biotechnology Reports 8:387-397, DOI 10.1007/s11816-014-0329-0 (FI 1,59)
- ✓ Kairúz Hernández-Díaz E, Pérez-Alonso N, Capote A, Pérez A, Jiménez E, Chong-Pérez B (2013) Concentración mínima letal de higromicina B en la formación de callos y multiplicación de brotes de *Digitalis purpurea* L. Biotecnología Vegetal 13:23-31
- ✓ Pérez-Alonso N, Capote A, Gerth A, Jiménez E (2012) Increased cardenolides production by elicitation of *Digitalis lanata* shoots cultured in temporary immersion systems. Plant Cell Tiss Organ Cult 110:153-162 (FI3,63)
- ✓ Pérez-Alonso N, Jiménez E (2011) Producción de metabolitos secundarios de plantas mediante el cultivo *in vitro*. Biotecnología vegetal 11:195-211
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